MICROBIOME ANALYSIS OF WHOLE SALIVA USING NEXT GENERATION SEQUENCING TECHNOLOGY IN IMPLANT SUPPORTED PROSTHESIS 6 MONTHS POST LOADING

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled,

"MICROBIOME ANALYSIS OF WHOLE SALIVA USING NEXT GENERATION SEQUENCING TECHNOLOGY IN IMPLANT SUPPORTED PROSTHESIS 6 MONTHS POST LOADING" is a bonafide and genuine research work carried out by me under the guidance of **Dr. B. SHIVAKUMAR, M.D.S.,** Professor, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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This dissertation is submitted to **THE TAMILNADU Dr.MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABRREVIATIONS

ABBREVIATION	EXPANSION
PSD	• Polymicrobial Synergy and Dysbiosis
DNA	• Deoxyribonucleic acid
Rrna	• Ribosomal Ribonucleic acid
HOMIM	• Human Oral Microbiome Identification Microarray
NGS	• Next Generation Sequencing
MSR	• MiSeq Reporter software
HOMD	• Human Oral Microbiome Database
HOMI	• Human Oral Microbiome Index
OUT	• Operational Taxonomic Unit
PCR	• Polymerase Chain Reaction
SoLiD	• Supported Oligonucleotide Ligation and Detection
BLAST	• Basic Local Alignment Search Tool
MiSeq	• Metagenomic Sequence

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Introduction

INTRODUCTION

The human body is estimated to be composed of more than 10^{14} cells of which only 10% of the entire composition is of mammalian origin. Microorganisms form the major composition of cells that are found in our body, forming a resident microflora, surviving via a symbiotic relationship with the host and thus maintaining homeostasis[7].

The oral cavity of the human body contains a number of different habitats, including the gingival sulcus, teeth, tongue, cheeks, hard and soft palates, and tonsils, which are colonized by bacteria. The oral microbiome is comprised of over 600 prevalent taxa at the species level, with distinct subsets predominating at different habitats, this was stated by **Floyd E. Dewhirst**. It displays the largest core of commonly related microbes among unrelated subjects when compared to the microflora found in the gut or on our skin. The relationship between periodontal microflora and the host is known to be benign, but changes in the salivary microbiome and bacterial community structures can tilt the scales and cause a disruption in the symbiotic relationship, thereby leading to the pathogenesis of periodontal disease.

Microbial shift, more commonly known as **dysbiosis**, refers to the concept that some diseases are due to a decrease in the number of beneficial symbionts and an increase in the number of pathogens[20]. The long-standing paradigm is that, as periodontitis develops, the oral microbiota shifts from one consisting primarily of gram-positive aerobes to one consisting primarily of gram-negative anaerobes [94].

Research over the past decade has led to recognition of microbes residing in various oral ecological niches as a part of dental plaque. Plaque biofilm is a highly organized accumulation of microbial communities adhering to favourable environmental surfaces, which functions to maximize energy, spatial arrangements, communication, and continuity of bacterial communities [38]. The origin, development and structural adaptation of dental plaque is governed by a dynamic, ever-changing equilibrium between oral microbiota and multiple factors that differentially promote or inhibit survival of its microbial constituents.

Based on the earlier studies conducted by **Socransky et al [83]**, the key bacterial species which play a role in the disease process have been segregated into microbial complexes based on their correlation with clinical parameters and severity of periodontal disease.

Currently, the pathogenesis of periodontal diseases is explained by "**Polymicrobial Synergy and Dysbiosis (PSD) Model**" proposed by **Hajishengallis et al** [20]. This model states that dysbiotic

environment and polymicrobial synergy are the key events that led to development of periodontitis rather than individual bacterial species.

Technological research led to advancements in DNA sequencing and bioinformatics tools which later led to the development of the Omics technologies. It was observed that every individual has a specific microbiome and a metagenome that is unique and specific to that person. The development of 16s rRNA and subsequently the Next Generation Sequencing technologies led to further characterization of the microbiome [57]

Human Oral Microbiome Database (HOMD) is software that currently lists the entireset of organisms present in the oral cavity as a whole. Several ecological niches have been identified within the oral cavity, each with its very own distinct microbiome [17].

Sampling fluids from the oral cavity, instead of other bodily fluids like blood or urine, provides an accessible medium in which a range of candidate biomarkers, such as electrolytes, proteins, antibodies, hormones and DNA/RNA, as well as other substances such as therapeutic drugs can be detected. While oral fluid assessment has been suggested to be useful in screening for or diagnosing oral or systemic diseases, monitoring viral or fungal infections, there is a lack when it comes to detecting drug exposure, and evaluating endocrine

disorders and cancer risk, evidence of utility for most of these purposes is lacking.

There continues to be interest in oral fluid as a diagnostic medium for rapid, point-of-care testing. Potential advantages of using saliva for disease diagnostics include the sample collection in toto being an easy and noninvasive procedure which thereby increases patient compliance, and there is a decrease in the risk of infectious disease transmission. Readily accessible fluids include: whole saliva, secretions from specific glands, mucosal transudate, or gingival crevicular fluid. Although various methods of collecting saliva and other oral fluids affect the precision and determination of biomarkers of interest, to date, there are no established uniform criteria available for the collection of human saliva [29, 35].

Dental implants are made of materials which are biocompatible with the host tissue, which when placed in the alveolar bone provide anchorage and support to the prosthetic superstructure that is fabricated. Peri-implant tissues are only thought to be of microbial origin and closely to parallel to periodontal diseases in the form of etiopathogenesis. However there is still some ambiguity in literature about the microflora in implants when compared to tooth.

Development of alternative methods instead of the traditional culturing methods has led to assess dental biofilms based on DNA analysis or other molecular techniques. Studies of salivary microbial community identified a link between taxonomic composition and disease pathogenesis. Current trend in sequencing of microbiome is based on Next-Generation Sequencing (NGS) that uses parallel sequencing of multiple small fragments of DNA to determine genetic sequences.

NGS technology was utilized earlier in our department (unpublished data) to identify and characterize subgingival microbiome from periodontal pockets and gingival recession.

Salivary microbial profiling may be a promising method of identifying further disease progression in both periodontitis and peri-implantitis. In this regard, the salivary microbiome in periodontal health and in healthy implant sites must first be established.

For a better understanding and in an attempt to analyze and characterise the salivary microbiome in periodontal health and in healthy implants six months post loading using Next Generation Sequencing Technology, the current study was undertaken.

Aim and Objectives

AIM AND OBJECTIVES

Aim:

To determine salivary microbiome using NGS technology in patients with implant supported prosthesis placed (6 months post loading).

Objectives:

1. To identify and characterize the salivary microbiome in patients with implant supported prosthesis placed (6 months post loading) using Next Generation Sequencing Technology.
2. To compare and analyze the salivary microbiome in sites of in patients with implant supported prosthesis placed (6 months post loading) with those of periodontally healthy controls.
3. To study species diversity in salivary microbiome, and characterize novel micro-organisms in in patients with implant supported prosthesis placed (6 months post loading).

Review of Literature

REVIEW OF LITERATURE

The human body is an entire living organism as a whole composed of various cells that combine together to form tissues which in turn lead to the formation of organs. Microorganisms are present all over our body and they contribute to the majority of cells that are found in our body, thereby forming the resident micro-flora and survive by having a symbiotic relationship with the host, thereby maintain homeostasis [41, 73].

The oral microbiome

The human body is estimated to have10^4 cells, out of which only 10% are of mammalian origin [70]. The oral microbiome is completey unique and distinctive due to its characteristic physical and biological abilities and the properties associated to each site despite the potential movement of micro-organisms between sites. They have the ability to attach to tooth surfaces or the epithelial surfaces of the gingiva and periodontal pocket. Combining these key observations led to the formulation of a core concept that illustrated the properties of the habitat are selective and dictate which organism are able to colonise, grow and whether they are minor or major members of the community [25, 93]

The Germ Theory

The **germ theory of disease** is the scientifically accepted current concept of disease. It states that, the main causes of many diseases are micro-organisms. These small organisms, microscopic in nature, invade the human cells, animal cells, and other living hosts. Their ability to grow and reproduce within the host cells can cause a disease. "**Germ**" doesn't just refer to bacterial origin but to any type of microorganism, especially one which causes disease, such as fungi, viruses, protozoa etc.

Microorganisms that cause diseases are classified under the term "PATHOGENS". The diseases they cause are of an infectious nature. Even when a pathogen is the principal cause of a disease, other factors like environmental and hereditary often influence the severity of the disease, and whether a potential host individual becomes infected when exposed to the pathogen.

The germ theory was proposed by **Girolamo Fracastoro**[33, 43, 123] in 1546, and expanded upon by **Marcus von Plenciz** [82] in 1762. Even though the theory was established, it was not popular among the practising doctors and scientists during the day, they still followed **Galen's** miasma theory [49]. The drawback of this belief in the theory prevented them from understanding how diseases actually progressed, with predictable consequences. By the early nineteenth century, smallpox vaccination was common among the practioners in Europe, though doctors were unaware of how it worked or how it's principles extended

to other diseases. Viruses were later discovered in the late 1800s sometime around 1890.

The Miasma theory

The miasma theory was the most common theory of disease followed in the 19 the century before the establishment of germ theory. It stated that diseases such as cholera, chlamydia infection, or the Black Death were caused by a *miasma* (Ancient Greek: "pollution"), a noxious form of "bad air" emanating from rotting organic matter. The theory posited that diseases were the product of environmental factors such as contaminated water, foul air, and poor hygienic conditions. Such infections, according to the theory, were not passed between individuals but would affect those within a locale that gave rise to such vapours.

Robert Koch and his Postulates

Robert Koch is known for develop Koch's postulates that demonstrated, in a scientifically sound manner, that a disease is usually caused by a particular organism. These postulates were formulated from his work with anthrax using purified cultures of the pathogen that had been isolated from diseased animals.

Koch's postulates were developed to serve as guidelines as to which pathogens can be isolated in the treatment of a disease in the 19th century. In the 1980s, a

molecular version of Koch's postulates was developed to guide the identification of microbial genes encoding virulence factors.

Koch's postulates:

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Socransky's criteria

Proposed a criteria by which periodontal microorganism can be classified as a periodontal pathogen based on checkerboard DNA technique, he later classified the different colour complexes of the bacteria based on their time of colonization[83, 106, 108]

1. Must be associated with disease, as evident by i**ncrease** in the **number of organisms** at diseased sites

2. Must be eliminated or decreased in sites that demonstrate **clinical resolution** of disease with treatment

3. Must demonstrate a **host response**, in the form of an alteration in the host cellular or humoral immune response.

4. Must be capable of **causing disease in experimental animal model.**

5. Must demonstrate **virulence factors** responsible for enabling the microorganism to cause destruction of periodontal tissue [109]

Dental Implants

Dental implants have become an indispensable and in vogue form of therapy in dentistry in order to replace missing teeth in different clinical situations. With success rates as high as 82,9% 16 years post loading have been reported. With careful attention to the indications, anatomical and also keeping in mind the intra-individual limiting factors, insertion of dental implants seems to represent itself as "safe" modality when it comes to the treatment option of replacing missing teeth [42, 43]. Nevertheless, in the last few decades increasing evidence have been raised on the presence of peri-implant inflammations [55, 57]. It is being represented as one of the most frequent complications affecting both the surrounding soft and hard tissues which can lead to the loss of the implant. Therefore, strategies for prevention and treatment of peri-implant disease should be integrated in modern rehabilitation concepts in dentistry [50].

Peri-implant Tissues and Peri-implant Diseases

The soft tissue surrounding a dental implant is termed as the peri-implant mucosa while the soft tissue surrounding a tooth is known as the gingiva. Although there are similarities between these two types of tissues, they have differences among each other present at a histological level [69]. The soft tissue present around an implant consists of keratinized and non-keratinized epithelium, similar to that of the periodontal tissues around the natural tooth. Unlike the natural tooth, there is an absence of the periodontal ligament between the implant and the surrounding bone, and the implant-bone contact is directly supplied, and is not done not via the fibers [104].

Because the dental implants placed in the oral cavity are in direct contact with the bone, the forces applied cannot be compensated. In addition, the peri-implant tissues are devoid of mechanoreceptors in the periodontal region that which are crucial when it comes to recognize the sense of touch.

Pathological changes that occur in the tissues that surround the implant are called peri-implant diseases. If these inflammatory changes are limited only to the soft tissues, they are known as peri-implant mucositis and they are called peri-implantitis only when the disease spreads to the underlying alveolar bone[2, 4]. In a systematic review published by **Derks et al. in 2015** [20], the prevalence of the condition called peri-implant mucositis was reported to be between 19% and 65%, and the prevalence of peri-implantitis ranged betweeen 1% to 47%.

Mucositis is a term that describes a bacteria-induced, reversible inflammatory process of the peri-implant soft tissue with reddening, swelling of the soft tissue and also bleeding on periodontal probing.

In contrast to mucositis, **peri-implantitis** is a more progressive and irreversible disease of the hard and soft tissues surrounding an impalnt and is accompanied usually with bone resorption and decreased osseointegration along with increased pocket formation and purulence. After the insertion of titanium implants, within the peri-implant sulcus, rapid colonization of bacteria has been observed[127]. Some microbiological studies have shown that implants affected by peri-implantitis tend to harbour microbiota consisting of the key periodontal pathogen species, including *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Tannerellaforsythia, Prevotella intermedia,* and *Fusobacterium* species.

Clinically, typical symptoms associated to peri-implant infections are: redness, oedema and bleeding observed in the soft tissues in the peri-implant mucositis. In peri-implantitis patients, clinical findings such as an increase in probing depth values and detection of radiographic bone loss are usually added to the already present clinical symptoms [14]. The etiology of peri-implant diseases and the etiology of periodontal diseases are similar. However, the fact that there is no real fibrous connective tissue attachment around the implants as it is in the teeth makes the tissues around the implant makes it more sensitive to microbial attack. Bacterial infection has a very important role in the success of

dental implants. In clinical and animal studies, bacterial plaque accumulation around the implant caused inflammatory reaction in peri-implant tissues.

Microbial etiology of Peri-implant diseases

Considering that the infectious nature of peri-implantitis is caused by the accumulation of a complex biofilm community on the implant surface, it seems sensible to try to map out the microbial profile of this disease. However, till date any hope of finding out what the actual cause of a peri-implant infection is still not a reality[17]. This has happened far earlier for periodontitis, and a lot of common lessons apply for both pathological entities. Therfore, the fundamental principle that is applicable for periodontitis can be used for peri-implantitis as well, i.e. peri-implantitis like periodontitis it is an opportunistic, endogenous, polymicrobial infection. This implies that the pathological species associated with the destructive disease are parts of the normal oral microbiota, but under certain ecological shifts (dysbiosis) become pathogens, never alone, but always acting in synchrony. It is not the mere presence of specific bacteria instigating the disease origin rather it's the interplay of divergent abundant bacteria from different phyla.

Over the years different methods have been applied to characterize the peri-implant microbiota. Culture-dependent methods have traditionally been the gold standard, but now molecular methods have been introduced to avoid conventional time-consuming laboratory work. With the new millennia, sequencing methods have started to take over the industry, including the latest

next-generation sequencing, have emerged to reveal in more detail differences between healthy and diseased oral microbiomes [17].

Bacterial colonization on the implant surface starts 30 min after insertion, and similar bacterial taxa can be identified on the implant after several months. The bacterial composition of the biofilm formed and observed on implants is similar to that of the adjacent teeth [30]. Hence, the microbial flora found on natural teeth are actually ***''a reservoir''*** for the biofilms that build-up around implants. In respect to the initial (i.e. 4 weeks) subgingival colonization, the frequency of detection of different species is similar between that of natural teeth and implants. Nevertheless, the colonization pattern on implants appears to be initially slower than on natural teeth. The peri-implant microflora in health consists mainly of Gram-positive cocci and non-motile bacilli, and a limited number Gram-negative anaerobic species, resembling gingival health. Nevertheless, the switch to peri-implant mucositis is associated with an increase in the presence of cocci, motile bacilli and spirochetes, at proportions comparable to gingivitis. The transition to peri-implantitis is associated mainly with the emergence of Gram-negative, motile, and anaerobic species that are commonly found in periodontits [52, 65].

Based on clinical studies it was observed that while the three ''red complex'' species, namely *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*, can be found at higher counts in peri-implantitis. Collectively, it appears that the qualitative composition of the biofilm microflora in peri-implantitis resembles that of periodontitis, this inturn

implies to the fact that patients with active periodontal disease are at higher risk for developing peri-implantitis. Submucosal biofilms obtained from peri-implantitis patients also yield bacteria that display in-vitro resistance to one or more standard antibiotic treatments. These are most often *Prevotella intermedia/nigres-cens* or *Streptococcus constellatus*.

Nevertheless, a number of microorganisms have been identified in peri-implantitis patients that are not commonly observed in patients with periodontitis [32]. These include bacterial species such as *Staphylococcus aureus, Staphylococcus epidermidis, Enterobacter aero-genes, Enterobacter cloace, Escherichia coli, Helicobacter pylori, Peptostreptococcus micra, Pseudomonas spp*, as well as *Candida spp* fungi. Presence of *S. aureus* shortly after implant insertion can be confirmed even one year later. It has also been shown that up-to 18.6% of peri-implantitis lesions harbour aerobic Gram-negative bacilli, such as enteric rods and coliforms, or non-enteric rods, but the microbial concentration may not fully correlate to the severity of the disease.

Putative periodontal pathogens may be detected at higher levels, prevalence and numbers, than in fully edentulous patients. Early studies indicated absence of detection of *Aggregatibacter* .

Higher roughness and **higher free energy** of the implant surface may favour biofilm formation, whereas peri-implantitis may occur earlier, with a faster and more extensive progression in implants with rougher surface.

In this respect, potential differences in bacterial adhesion due to surface microstructure may partially be equilibrated by the mediating salivary pellicle.

Hence, given the biological involvement of the pellicle, implant surface characteristics may not notably affect the initial stages of biofilm formation and composition.

Peri-implant Tissue Microbiology

Peri-implant diseases after successful osseointegration of intra-bone implants are caused by an imbalance between bacterial activity and host response. The response to inflammation in peri-implant diseases may be confined to the mucosa around the implant as it is in the peri-implant mucositis, or it may progress and cause loss of bone tissue and lead to peri-implantitis[72].

Periodontal and peri-implant diseases are infections caused by microorganisms. Therefore, the use of microbiological parameters has an important role in both microbial development and routine diagnosis and follow-up. It has been reported that periodontal pathogens are detectable around dental implants that begin to function in the oral environment in a short period of a month[79].

After implant placement perioodontopathogens are found present in healthy microflora. **Koka et al**.[36] in his study observed the existence of *P. gingivalis, P. intermedia, Actinomyces naeslundii, F. Nucleatum* and *T. denticola* in the

plaque samples on the 14th and 28th days after opening of the implant into the oral environment was investigated in comparison with natural teeth.

According to the data that was obtained, these species can be determined after 14 days and within 28 days; a complex salivary microbiota has been reported to colonise the site . Although there are only a few changes in the microbial species profile around the tooth, there has been a significant increase in the amount of *F. nucleatumss vincentii, Peptostreptococcus micros, Prevotella nigresence* and *P. Gingivalis* species around the implant over time. The species such as *S. mitis* and *S. oralis* seen in early colonization on teeth were also colonized at the second week around the implants and maintained their levels for 26 weeks. It has been reported that in the salivary flora of clinically healthy implants, there is a high proportion of bacteri a in the form of cocci, and that spirochetes, motile bacilli and Gram (-) anaerobic species are none or low. It has been reported that there is a high rate of spirochetes and motile bacteria in clinically deep pockets and implants with increased alveolar bone loss [79].

Periodontopathogenic bacteria such as *porphyromonas gingivalis, treponama denticola, tannerella forsythensis* and *aggregatibacter actinomicetocomitans* have been shown to be similar in peri-implant pockets and in periodontal pockets.

Leonhardt et al.[39, 41]compared microbial flora found in the vicinity of healthy implants with the one in implants where peri-implantitis observed, they reported that in the study group 60% of the implants were detected with *P.*

gingivalis, P. intermedia, A. actinomycetemcomitans and *P.nigressence* with 55% *Staphylococcus spp., enteric* and *Candida spp*. and none of these microorganisms were detected in the healthy group.

Implant loss can be differentiated on the basis of the following additional factors:

- Overloading of the implant,
- Faults in material and techniques,
- Poor bone quality at the implant area,
- Systemic diseases and drug therapies, which inhibit bone modulations according to "Wolff's law" (bone density and strength increase with stress - and vice versa).

Thus, implants of more than 10 mm length in square thread design show higher success rates than shorter implant lengths or shapes without thread or buttress thread [62, 65]. Also rough implant surfaces of more than 2 microns seem to feature better osseointegration than smooth (<0.5 microns) or moderate surfaces (1–2 microns) [105]

Mechanical overload in implants

Occlusal trauma in an implant patient may be defined as an injury to apparatus used as an attachment is the result of excessive occlusal force directed to the apparatus. There is a current controversy regarding the role of occlusion in respect to the amount of bone loss observed after the delivery of animplant prosthesis [112].

There is generalized consensus that was made in respect to the fact that early implant failure may be associated with overload. However, some articles conclude that peri-implant bone loss without implant failure is primarily associated with biological formations or complications [1, 48].

Other authors suggest a correlation of crestal bone loss to occlusal overload.

The relationship between "***stress and strain***" determines the modulus of elasticity (stiffness) of a material [67]. Hence, the modulus refers to the amount of dimensional changes that occur in a material for the given amount stress level. The ***modulus of elasticity*** of a tooth is similar to that of cortical bone. Dental implants are usually fabricated using titanium or its alloy. The modulus of elasticity of titanium is 5 to 10 times greater than that of cortical bone [5, 8].

An engineering principle known as "***the composite beam analysis***" states that when two materials of different elastic moduli are placed together with no intervening material, of which one is loaded, a stress contour increase will be observed where the two materials first come into contact.

In the oral cavity when it comes to an implant-bone interface these said stress contours are of greater magnitude at the bone's crestal region [90]. This phenomenon was observed in both photo-elastic and also a 3-dimensional finite element analysis studies when implants were loaded within a bone simulant.

Bone Mechanical Properties

The density of bone is said to be directly related to the strength and elastic modulus of bone. Hence, in bone with higher density the amount of strain is lesser and experiences a lesser load when compared to softer bone [68]. As a result, the amount of remodeling in denser boner is lesser compared to softer bone when they experience similar load conditions [3].

A decrease in the amount bone remodeling can result in lower levels of bone loss. In a prospective human study, **Manz** observed that the amount of marginal bone loss observed next to an implant was directly related to the density of the bone.

Hoshaw et al conducted a study in dogs where loaded threaded implants were placed in the oral cavity of the canines with a tensile load and he noted that the fine trabecular bone pattern became coarse trabecular bone around the implant [98]. Fine trabecular bone is less dense when compared to coarse trabecular bone. Since the density of bone is directly related to its strength and elastic modulus, the crestal bone strength and biomechanical mismatch between titanium and bone may improve in relation to the functional loading. In other words, the stresses applied to the peri-implant bone may be great enough to cause bone resorption during the first year, since bone strains are greatest at the crest. But the stress applied below the crest of bone is of less magnitude and may correspond to the physiologic strain, which allows the bone to gain density and strength [103]. As a result, the occlusal load that causes bone loss initially (overload), is not great enough to cause continued bone loss once the bone matures and becomes more dense.

Implant Design Biomechanics

Implant design may change the amount or type of forces applied to the bone-implant interface[118]. A smooth collar at the crest module may transmit shear forces to the bone. Bone is observed to be strongest under compressive forces, 30% weaker under tensile loads, and 65% weaker to shear forces.

Bone may heal to the smooth metal collar of the implant crest module from implant insertion to implant exposure; but when under loading conditions, the weaker shear interface may be more inclined to overload the bone. The first thread of the implant is where the type of force changes from primarily shears to compressive and/or tensile loads.

Therefore, in many situations the 35 to 65% increase in bone strength through changes from shear to compressive loads is sufficient to halt the bone loss process. This may be one of the reasons why implant designs with a 2 mm smooth collar above the first thread and a 4 mm smooth collar above the first thread, both lose bone to this "first thread" landmark [12, 113].

Factors of the implant affecting adhesion of bacteria on its surface

Surface roughness

Broad and detailed research shows that both the amount of plaque formation and the maturity of the plaque, with increasing numbers of motile rods,

increase in proportion to the roughness of the surface. Several studies have investigated roughness and bacterial adhesion by altering a titanium surface.

According to a study conducted by **Amoroso et al**, the adhesion of *Porphyromonas gingivalis,* as a cause of periodontal disease, significantly declined on a "very smooth" titanium surface, i.e., much smoother than the one commonly used as an implant abutment (Ra = 34.57 vs. 350 nm, respectively) [6,119].

The dependence of bacterial adhesion on titanium surface roughness was confirmed in a recent *in vitro* study.

Among three titanium disc surfaces, an acid-etched and blasted surface showed significantly higher roughness and proportionately higher adhesion by *Streptococcus mutans, Porphyromonas gingivalis*, and *Fusobacterium nucleatum* than either a machined or acid-etched surface.

A recent study using an *in vivo* model evaluated the effects of titanium surface roughness on initial bacterial adhesion by *Streptococcus sanguinis*, *Actinomyces naeslundii*, and *Lactobacillus salivarius*. The rougher blasted surface, with a Sa of about 1.5 μm, showed greater bacterial adhesion than the turned surface, with an Sa of 0.18 μm. An anodicallyoxidized surface (Sa = 0.4 μm) also promoted greater microbial attachment than the turned surface. The augmented resistance of the rougher surfaces to shear forces was suggested to cause the increased bacterial adhesion [122, 135].

While bacterial adhesion declines as surface roughness decreases, there is a lower limit to this relationship, at a roughness called the "***threshold Ra***." **Bollen et al** reported that there was no effect on the salivary microorganism

composition in the short-term or in the long-term when the Ra was < 0.2 μm . In that study, the authors connected the titanium abutment (Ra = 0.2 μm) to the fixture and sufficiently grounded the ceramic abutment (Ra = 0.06 μm) also to the fixture. After intraoral exposure of these setups for 3 and 12 months, the clinical periodontal index and plaque samples were compared. Both the number and the composition of the pathogenic bacteria were found to depend on the roughness of the abutments, with an increase in probing depth and greater bleeding in response to probing determined on rougher versus the smoothest abutments [7].

This result correalated with the results of an *in-vivo* study by **Quirynen et al** [28], who monitored the clinical and microbiological findings obtained with four grounded titanium abutments, with Ra values ≤ 0.2 μm, for 3 months. While spirochetes were observed only around the roughest of abutments, there were no other differences observed in salivary microbial population, providing further evidence of a said threshold level below which reduced bacterial adhesion doesn't confer a clinical benefit [13]. Also, the results of this study showed that although some attachment gain (0.2 mm) was achieved in the roughest abutment, the other abutments had at least a minimum of 0.8 mm of attachment loss, indicating that a certain degree of roughness of surface is needed for providing a resistance against probing.

Surface free energy (SFE)

The technique called **sessile drop** is frequently used to determine the energy of solid surfaces. It involves the measurement of the contact angle between a droplet of liquid with a known surface energy and the solid surface of interest.

Because roughness is one of the several factors affecting the contact angle, roughness itself will affect the surface free energy (SFE).

Busscher et al reported the surface roughness's effects on the contact angle disappeared when Ra was < 0.1 μm. Thus, while the SFE is independent of roughness below certain values of Ra, further experiments must be conducted to interpret this finding.

Recent *in vitro* research has evaluated SFE and bacterial adhesion using disc samples whose surfaces consisted of a polished, partially stabilized zirconia material, (titanium blasted with zirconia, titanium blasted with zirconia, and then acidetched, or polished titanium). The surfaces of polished partially-stabilized zirconia and titanium blasted with zirconia were found to have a lower SFE and decreased bacterial adhesion property (*Streptococcus mitis* and *Prevotella nigrescens*). The authors concluded that SFE is the most important factor determining initial bacterial adhesion.

Bacterial adhesion was shown to correlate with the total SFE and the proportion of the nonpolar component of the material. These findings correlated with those of **Pereni et al**[73, 133], in which an association between SFE and bacterial retention was demonstrated, albeit using other bacterial species.

The SFE of the substratum is related to the SFE of the bacterial clusters. On low-SFE surfaces, bacterial clusters with lower SFEs were shown to be predominant. In addition to the SFE of the substrate and of that of the bacteria, that of the suspending medium is also important. In addition, the pellicle

coating was shown to have homogenizing effects on the SFE, indicating the complexity of SFE effects even under defined conditions.

MATERIALS

Titanium is commonly used as the abutment material because of its superior biocompatibility. Recently, however, zirconia has been increasingly preferred for esthetic reasons; thus, many studies have compared zirconia and titanium. The results of a study conducted by **Scarano et al** supported the use of zirconia. In that *in vivo* human study, an intraoral device adhered with either zirconia or titanium disc samples was exposed for 24 hours, after which the surface was analyzed with SEM to measure the rate of bacterial covering [20, 59]. Significantly less adhesion was observed with zirconia (12.1%) than with titanium (19.3%), indicating the appropriateness of the former as an abutment material.

Nonetheless, many researchers reported no differences between the two materials. **Rasperini et al** conducted a study in which a microbiological analysis of samples collected from titanium and zirconia abutments at 6 hours, 24 hours, 7 days, and 14 days were done [60,126]. Maximum colonization occurred after 24 hours of intraoral exposure and was maintained consistently until the 14th day, with no differences between the two materials. A similar study conducted by **Brakel et al** prolonged the observation period. Bacterial

composition and soft-tissue health at the second post-operative week and third post-operative month were not significantly different in the zirconia versus the titanium group. In an *in- vitro* study comparing pellicle composition and bacterial binding properties, zirconia and titanium yielded similar results that were significantly different from those obtained with hydroxyapatite[76].

A recent *in vivo* study compared dental ceramics with respect to biofilm formation. Glass ceramic, lithium disilicate glass ceramic, yttrium-stabilized zirconia (Y-TZP), pressed Y-TZP ceramic, and a pressed mixed ceramic with Y-TZP and 25% alumina, all with similar surface roughness (mean Ra = 0.04 μm), were tested. Plaque accumulation was lowest in the pressed Y-TZP ceramic and highest in the lithium disilicate glass-ceramic, suggesting that the material itself also has an effect on biofilm formation, although this is partly related to its surface energy [51, 133]. The use of a gold alloy as an abutment material analogous to the use of zirconia and titanium has been examined in several animal and clinical studies aimed at estimating its biological reliability by measuring the periodontal index and assessing soft-tissue stability.

Salivary diagnostics

Saliva and other oral fluids (for example: oral mucosal cellular secretions, and gingival creviculaxr fluid) support the health of the soft and hard tissues present in the oral cavity. The protective functions of saliva mainly include maintaining a neutral oral pH, cleaning and re-mineralizing teeth, facilitating

swallowing and digestion, and protecting oral tissue against desiccation and invasion by micro-organisms[76, 86].

Adequate saliva is essential for maintaining oral health, and reduced salivary secretion (i.e., hyposalivation) or xerostomia (i.e., dry mouth) can contribute to oral problems such as dental-caries, periodontal diseases,etc [10]. There continues to be interest in oral fluid as a diagnostic medium for rapid, point-of-care testing. Potential advantages of using saliva for disease diagnostics include ease of access, noninvasive sample collection, increased acceptance by patients, and reduced risks of infectious disease transmission. Readily accessible fluids include whole saliva, secretions from specific glands, mucosal transudate, or gingival crevicular fluid[36, 39].

Although various methods of collecting saliva and other oral fluids affect the precision and determination of biomarkers of interest, to date, there are no established uniform criteria for collection of human saliva[19, 67]. Currently, oral fluid testing by clinical laboratories is regulated under the Clinical Laboratory Improvement Amendments of 1988 (CLIA).

Oral Fluid Bio-components

Bio-component Class	**Examples**
Hormones	Cortisol, androgens, estriol, estrogen, progesterone,

	aldosterone, melatonin, insulin
Cytokines	Interleukins (IL-1beta, IL-6, IL-8), tumor necrosis factor, troponin
Antibodies	IgG, IgA
Proteins/Enzymes	Amylase, pepsin, matrix metalloproteinases, C-reactive protein (CRP), mucins, lactoferrin, antimicrobial peptides
Growth Factors	Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) insulin-like growth factor
Nucleic Acids	Human and microbial DNA, mRNA, microRNA, tRNA-derived small RNA (sRNA)
Viruses	HIV, HSV-1, HSV-2, EBV, HPV, CMV, VZV, HCV
Bacteria	*P. gingivalis, S. mutans, Lactobacillus spp, T. forsythia, E. coli, H. pylori, M. tuberculosis*
Fungi	*Candida, Aspergillus*
Drugs	Anticonvulsants, chemotherapeutic agents (including antibiotics and antineoplastic agents), analgesics, drugs of abuse, ethanol
Metabolites/Electrolytes	Phosphate, calcium, sodium, potassium, glucose, chloride, nitrate, uric acid, amino acids, lipids, carbohydrates

Tumour Markers	CA 15-3, HER2/neu, CA 19-9, p53, leptin, CA 125, alpha fetoprotein, CEA, somatic mutations in tumor suppressor genes, loss of heterozygosity, promoter hypermethylation of genes, microsatellite DNA alterations

Peri-implant sulcular fluid (PISF)

The current criteria used in the diagnosis of periodontal conditions clinically, has created a limitation in the utility to predict disease progression in the future [20]. The potential role of host response and microbial biomarkers obtained from oral fluids have been investigated and based on these investigations they are being used as complementary diagnostic tools for periodontal disease. Concentrations of host response molecules maybe used as a representation for a more accurate measurement of real-time disease activity than conventional clinical measurements [28,37]

Microbial communities from subgingival and supragingival biofilm have been clustered in complexes according by their relationship to commonly used clinical parameters [83,105].

The red complex bacteria (*Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola*) and orange complex member (*Prevotella intermedia*) have been pointed out as the species responsible for chronic periodontitis [37], while *Aggregatibacter actinomycetemcomitans* for aggressive

periodontitis [47]. Furthermore, besides being highly associated with disease, assessments of microbiological presence and load are able to predict its progression in susceptible sites [9, 53]The pairing of both data has been proven valuable to increase their diagnostic abilities in crosssectional and longitudinal models in subjects afflicted with periodontitis [36, 54]**.** Conversely, limited information regarding the usefulness of biomarkers upon peri-implantitis is available.

Radiographic and clinical assessments such as radiographic bone loss evaluation, peri-implant probing, bleeding on probing (BOP), microbial testing, implant mobility, and suppuration all serve as traditional measuring tools for peri-implant surveillance and disease diagnosis [86]. Moreover, an increasing interest for the assessment of numerous inflammatory mediators, host proteolytic enzymes and tissue breakdown biomarkers, in addition to angiogenic and matrix metalloproteinase inhibitors biomarkers within gingival crevicular fluid (GCF) and peri-implant crevicular fluid (PICF) has been targeted for disease detection and prediction to elucidate a broad overview of the different phases of the periodontal and peri-implant diseases [87, 132]

Identifying a single predictive biomarker for periodontal and peri-implant diseases would be of great significance. However, microbial profiles and a combination of several host response biomarkers around dental implants could reveal a more precise assessment of a disease status than traditional clinical measurements as observed in a periodontitis model [35, 36]

Factors that correlate with marginal bone loss in implants

A positive correlation has been found between oral hygiene and marginal bone loss around implants in the edentulous mandible [48]. During peri-implant breakdown a complex microbiota is established, closely resembling that found in adult periodontitis [31, 77]. When peri-implant tissue breakdown is induced by placing plaque retentive ligatures submarginally, a shift in the microflora occurs [64, 65]. Apart from darkpigmented Gram-negative anaerobic rods, other bacterial species associated with per-implant infection include *Bacteroides forsythus*, *Fusobacterium nucleatum*, *Camphylobacter*, *Peptostreptococcus micros*, and *Streptococcus intermedius* [58]. Organisms not primarily associated with periodontitis, such as *Staphylococcus* spp., Enterics and *Candida* spp. have also been found in peri-implant infection) [87].

Association of peri-implant disease with periodontal disease

Studies have shown that the microbiome that is found around implants that have failed are similar to that found around the teeth affected with periodontitis in their composition as well as their proportion of microorganisms [100,116]. Hence, the association of peri-implant disease with periodontal disease have been reported for several studies.

The incidence of the condition peri-implantitis has increased continuously over the last few years, ultimately relating it with the loss of implants. Many factors play a fundamental role in creating complications that are related to implant failures like the implant and prosthetic component's design as well as bacterial colonization around implants [4]. The colonization of bacteria around dental implants can lead to some local diseases, the most common being:

mucositis and peri-implantitis. These two forms of infections resemble gingivitis and periodontitis respectively.

Based on a study conducted by **Renvert & Persson**, showed that patients with a previous history of periodontitis have been proven to have an increased risk of acquiring peri-implant infections. Another study conducted by **Heitz-Mayfield & Lang** [64], concluded that patients who are susceptible to periodontal disease, are seen to possess a higher susceptibility to peri-implant disease when compared to those patients without any history of periodontal disease. To decrease the chances of implant loss due to the presence of periodontopathogenic bacteria, several researchers have proposed the elimination of these pathogens before dental implant installation.

Although, the microbiological profile at failing implants differs from those in peri-implant health, it is not known whether the host response observed in peri-implantitis patients is similar to that seen in periodontitis [117].

Since a great number of patients receiving dental implants are patients with a history of periodontal disease, i.e. individuals who previously have shown a tissue destructive inflammatory response, a microbial challenge in conjunction with an aberrant host response are possible etiologic factors.

Analysis of inflammatory mediators in crevicular fluid has also been used to compare peri-implant tissue health and disease. Neutrophil- derived enzymes such as neutral protease, neutrophil elastase, myeloperoxidase and b-glucuronidase have been found in association with failing implant sites [11]. Interleukin-1 (IL-1) is a potent proinflammatory cytokine that can influence the host response in the inflammatory tissue destruction. IL-1 is produced

mainly by macrophages but also by other cells including neurtrophilic granulocytes [92].

Peri-implantitis may have a multifactorial background where an aberrant host response in conjunction with the bacterial challenge may contribute to the development of tissue destruction around implants. Elucidation of factors of importance for peri-implant tissue destruction should make it easier to predict which patient or implant is at risk for peri-implant complications during maintenance and retention of implants. In the present study, we therefore aimed to characterise markers of inflammation, microbiota and inflammatory host response at implants and teeth in patients with signs of marginal tissue destruction, i.e. peri-implantitis [107,110]

Salivary diagnostics and implant patients

The current world of research is probing the different facets involved in periodontal disease diagnostics and is currently investigating the possible use of oral fluids, such as saliva, for disease assessment. The secretions obtained from the major salivary glands (parotid, submandibular and sublingual), which usually consist of a large number of proteins and peptides, are responsible for maintaining the integrity of the oral cavity[101, 121]. Also, because of its importance in oral biofilm formation and host defense, secreted saliva may play a significant role in the establishment and progression of periodontal disease.

Saliva an oral fluid is considered to be a mirror of the body. It could be used to monitor the general health and also the onset of specific diseases. Biomarkers, whether produced by normal healthy individuals or by individuals affected by specific systemic diseases, are tell-tale molecules that could be used to monitor health status, disease onset, treatment response and outcome. Informative biomarkers can further serve as early sentinels of disease, and this has been considered as the most promising alternative to classic environmental epidemiology[71, 130]

The salivary microbiota is a potential diagnostic indicator of several diseases. Culture-independent techniques are required to study the salivary microbial community since many of its members have not been cultivated.

The study of the oral microbiota as well as its salivary component requires culture-independent techniques, since about one third of 700 bacterial species identified in the human oral cavity have not been cultivated . These may be based on PCR amplification and high-throughput sequencing of the bacterial 16S rRNA genes (16S-HTS) or the metagenomic whole genome shotgun (WGS) sequencing [39, 57]. The latter approach may include either the analysis of the totality of generated DNA fragments or of the 16S rRNA gene fragments retrieved from the metagenome (16S-WGS). Both 16S-WGS and 16S-HTS approach presents limitations and advantages over each other [3, 84].

Salivary proteome: human salivary proteome project

Human salivary proteome analysis is of importance, as it helps us understand oral health in general and the pathogenesis of a disease. Three research groups

were funded by the National Institute of Dental and Craniofacial Research / National Institutes of Health to decipher comprehensively the human salivary proteome. Significant progress has been made in cataloguing human saliva proteins and exploring their post-translational modifications [78].

By using both two-dimensional gel electrophoresis / mass spectrometry and 'shotgun' proteomics approaches, we identified 309 distinct proteins in human whole saliva. In addition, after 3 years of collective identification and cataloguing of the salivary proteome by the three National Institute of Dental and Craniofacial Research-support salivary proteome projects, the first complete profile of the salivary secretory proteome has been completes. Collectively, 1166 salivary proteins have been identified: 914 from the parotid fluid and 917 from the combined submandibular and sublingual fluids [30]. The University of California at Los Angeles is the data-centralization site, harbouring the entire database of the human salivary proteome known as the 'Salivary Proteome Knowledge Base' is a genome-wide view of the distribution of the parotid and combined submandibular and sublingual fluid proteomes [44, 62].

Salivary transcriptome

Based on earlier studies it was found that RNA molecules are elevated in the cases of oral cancer tissues and their levels in saliva are also high, which also prompted to the examination of the scope and complexity of RNA present in human saliva. High-density oligonucleotide microarrays (Affymetrix HG U133A) were used to profile salivary mRNA and revealed that there are ~3,000 human mRNAs in the cell-free saliva supernatant of healthy subjects.

Of particular interest is that there is a normal saliva transcriptome core signature of 185 mRNAs that is present in all normal subjects, providing the rationale to use the salivary transcriptome for disease detection [34, 80].

The presence of human mRNA in saliva may seem to be surprising. However, it was shown that endogenous mRNA is protected from immediate degradation in a similar manner as cell-free RNA in plasma. The utilization of cell-free RNA has been widely accepted. Two recent studies demonstrated that foetal cell-free RNA crossed the placenta and was detected in maternal serum. The first study involved the non-invasive determination of foetal aneuploidies from maternal plasma [99]. The second study was a gene-expression microarray investigation of maternal and foetal whole blood that identified foetal mRNA markers in maternal blood that are independent of gender or polymorphism [131].

Other research groups, particularly from forensic sciences, are focusing on multiplex mRNA profiling for the identification of body fluids, including saliva. Most recently a Dutch forensic group was able to perform a gene-expression profile on saliva stains from crime scenes, leading to the identification of five saliva RNA markers (SPRR3, SPRR1A, KRT4, KRT6A and KRT13), stable for up to 180 days, which can be used for the identification of blood and saliva stains in forensic practice. Of importance is that these five saliva RNA markers selected for forensic applications are all in the normal saliva transcriptome core of 185 mRNAs, substantiating and independently validating our data. Lastly, **Shaw** and co-workers have identified a biomarker, amylase, which is highly correlated with sleep drive.

Importantly, both salivary amylase activity and mRNA levels are also responsive to extended waking in humans. These studies provide firm support for our work by showing that mRNA can be extracted from human saliva and used to develop standard tests [46, 111].

Immunologic and enzymatic assays

Enzyme-linked immunosorbent assay (ELISA) and Immunofluorescence techniques are basically antibody-based methods used to enumerate the specific species of microorganisms without actually cultivating the species. It is dependent on the specificity of developed antibodies to specific taxa. These techniques have the advantage that samples don't necessarily have to be cultured for their enumeration; they are rapid and less expensive than the method which involves a culture. However, they are limited to species for which reagents have been developed. It is difficult to use these techniques to evaluate species in large numbers of plaque samples and it is time-consuming to develop and validate specific antisera to new species [61, 134].

DNA – DNA hybridization or checkerboard

DNA-DNA hybridization is a molecular approach used to detect bacteria based on the hybridization of the target species to the ones that are the labelled genomic DNA that has been attached to nylon membranes. Based on extensive research and studies conducted by **LoescheWJ et al, Haffajee** [83]**Ximenez-Fyvie LA, Feres M et al**[118] and **Socransky** [104] have reported that the levels of limited number of species with this method is found in adult periodontitis,

periodontal health, refractory periodontitis and response to therapy. This method provides a major benefit for studies conducted on the oral microbial ecology due to advantages such as detection of multiple species from each sample simultaneously, and enabling the ease of study when it comes to a large sample size for large numbers of species [136]. Checkerboard technique is a rapid, sensitive, and relatively inexpensive technique. It is also dependent on a culture technique to cultivate the target species for creating genomic probes. Like antibody-based assays, cross reactivity can be verified only with cultivated species hence specificity of the probe is an unknown variable.

Polymerase chain reaction

Kary Mullis first developed the technique of polymerase chain reaction (PCR) , which was used to amplify specific genes or parts of genes which are then used to identify bacterial species from which they had originally originated from [61]. In a study conducted by **Kumar PS et al (2005**) [24, 66] species-specific PCR primers were designed and used in individual PCR reactions to detect prevalence of target species in plaque samples of healthy and diseased subjects. These studies confirmed that several species, including the ones that were uncultivated, were associated with oral health or periodontitis [21, 89]. Given the appropriate primers, this method is of a rapid, simple nature, can detect small numbers of cells of a given species, and indicates the presence or absence of a species in the sample. It has certain disadvantages of not providing data, and it may not be cost effective for a larger sample size and for applications where relative levels of species are important, PCR may not be ideal.

DNA Probes

Oligonucleotide probes are short probes that are designed to identify unique regions of DNA within cells of a given bacterial species. These probes are highly specific and the likelihood of cross-reactions with other species is extremely low [129]. Because they usually target a limited segment of DNA of an organism, oligonucleotide probes tend to be less sensitive for detection of low numbers of bacteria than whole genomic probes.

Whole genomic DNA probes are constructed utilizing the entire genome of a bacterial species as the target and therefore can be quite sensitive. This in turn may increase the probability of cross-reactions between species because of common regions of DNA among closely related species [45]. The technique can detect only species for which DNA probes have been prepared, creating a drawback as it cannot detect the novel pathogens or environmentally important species that might be detected in culture or by other molecular techniques.

OPEN ENDED APPROACHES- 16S rRNA sequencing analysis:

Open ended approaches usually allow identification of even uncultivated and previously unknown species. According to **Spratt (2004)** these approaches are based on the 16 S rRNA sequencing, it's amplification and resulting analysis of the 16S rRNA genes present within a microbiome sample. 16S rRNA has proven to be the most useful phylogenetic marker to identify bacteria and in turn determine their evolutionary relationships [23, 102]. Ribosomal RNA gene is essential for life and present in all prokaryotes. It contains nucleic acid sequences with variable and highly conserved regions;

these conserved regions are used to design universal PCR primers which are capable of recognizing segments of 16S rRNA gene sequence of all bacterial species and hypervariable regions are used as signatures to discriminate one species from another. 16S rRNA gene is large enough (about 1500 bases) to provide sufficient sequence variability among bacteria, thereby making comparisons possible at different taxonomic levels [39].

DNA and protein sequencing started in the 1970s when the virus Lambda (50,000 nucleotides) was sequenced by **Sanger et al. Frederick Sanger** and colleagues. They described the use of chain-terminating dideoxynucleotide analogues that caused base-specific termination of primed DNA synthesis and this came to be popularly known as Sanger sequencing method. This method of sequencing was then considered to be of gold standard, and over the years, whole genome sequencing of many bacteria has been carried out using this method. Sanger technology was used in the sequencing of the first human genome, which was completed in 2003 through the ***Human Genome Project***, a 13-year effort with an estimated cost of 2.7 billion dollars [74]

Over the past decade, next generation sequencing technologies have emerged, which are high throughput and they are able to generate three to four orders of magnitude more sequences and are also relatively less expensive.

NEXT GENERATION SEQUENCING TECHNOLOGY (NGS):

Next generation sequencing method employs a wide spectrum of technologies such as: sequencing by ligation, sequencing by synthesis, single molecule DNA sequencing and colony sequencing. NGS is performed by repeated cycles of polymerase-mediated nucleotide extensions or by machinery automated cyclical ligation of oligonucleotides [7].

Fundamentals of NGS platforms:

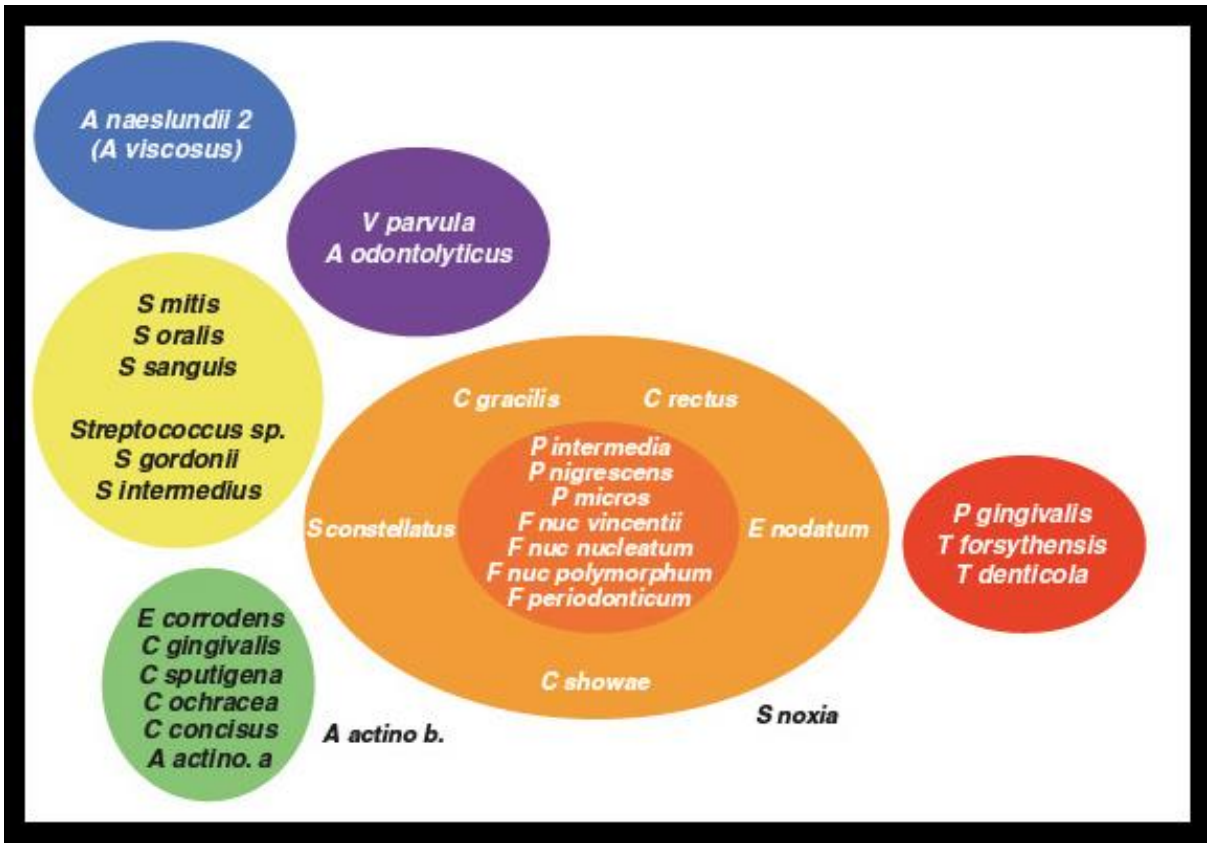
NGS platforms share a common technological feature—massive parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell[7, 115]. This design is a paradigm shift from that of Sanger sequencing, which is based on electrophoretic separation of chain-termination products produced in individual sequencing reactions. In NGS, sequencing is performed by repeated cycles of polymerase-mediated nucleotide extensions or, in one format, by iterative cycles of oligonucleotide ligation. As a massively parallel process, NGS generates hundreds of megabases to gigabases of nucleotide sequence output in a single instrument run, depending on the platform. The 2 basic procedures are ligation of DNA fragments with oligonucleotide adaptors and fragment immobilization to a solid surface, such as a bead [65, 88].

The three commonly used platforms for massive parallel DNA sequencing at present are the Roche/454 FLX, the Illumina/ Solexa Genome Analyze [34,75] and the Applied Biosystems / SOLiD (Life Technologies,

Carlsbad, CA). The most recent powerful NGS platforms have significant reductions in the run time and remarkable data output, they include HiSeq and the Ion Torrent Personal Genome Machine (PGM).

Until recently, the main focus in dental research has been focused on studying a small fraction of the oral microbiome which mainly consisted of opportunistic pathogens. After the advent of next-generation sequencing (NGS) technologies, researchers now have the tools that allow for profiling of the microbiomes and metagenomes at never before thought of depths thereby, creating a massive opportunity to understand the oral microbiome as a whole, better [91]. The major advantages of NGS are the high throughput and the fact that there is no need to target specific taxa. The availability of sequencing facilities and the relatively low cost have contributed to an exponential growth of NGS datasets. Using this technology we have conducted this study, to identify the microbiome associated with implant patients thereby establishing a sound knowledge as to the key colonisers which can further help us identify and rectify any future implant related diseases .

FIGURE 1: BACTERIAL COMPLEXES



Bacterial species	Complex
Actinomyces *Veillonella*	Purple
Streptococcus: gordonii, intermedius, mitis, sanguis	Yellow
Capnocytophaga *E. corrodens*	Green
Campilobacter rectus *Fusobacterium nucleatum* *P. micros* *P. intermedia*	Orange
T. forsythia *P. gingivalis* *T. denticola*	Red
A. actinomycetemcomitans *Selenomonas*	Not grouped

FIGURE 2: ROCHE 454 LIFE SCIENCES SYSTEM

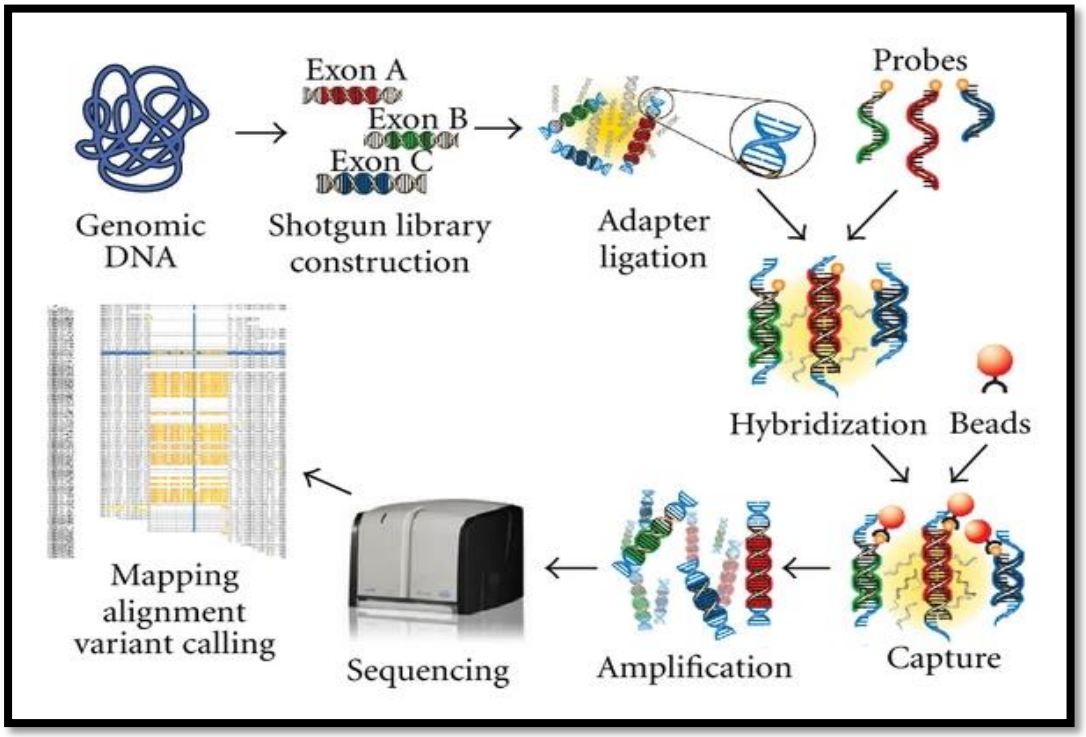
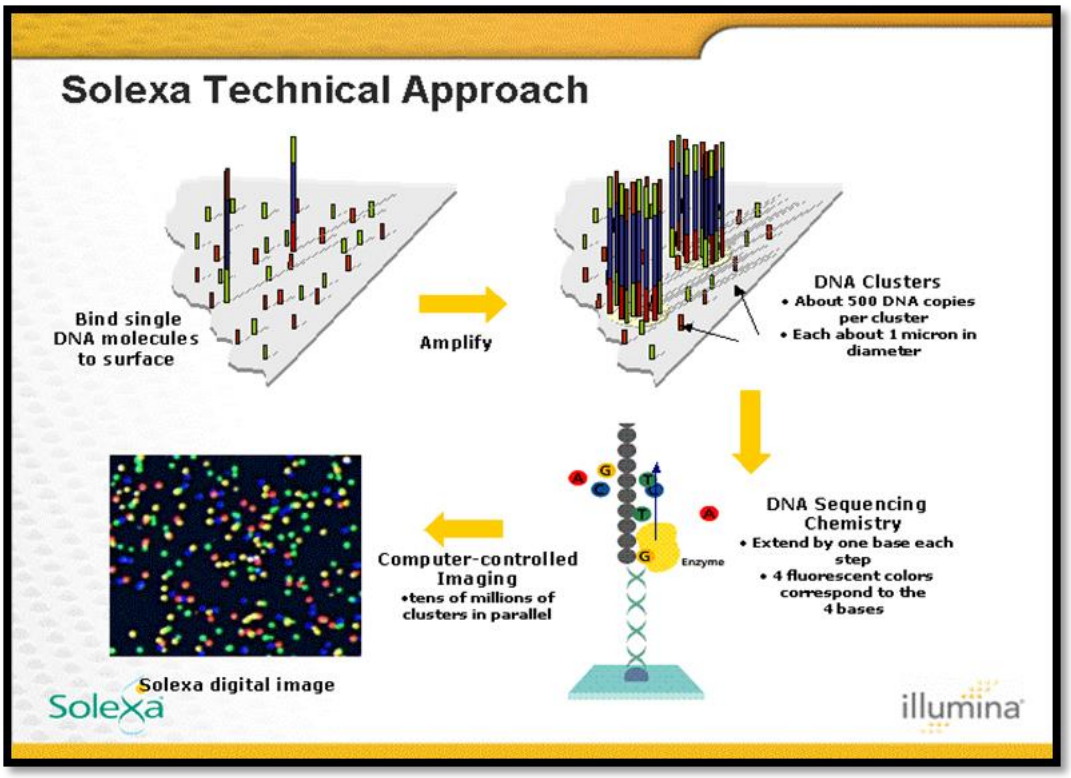


FIGURE 3: ILLUMINA SOLEXA GENOME ANALYZER



Materials and Methods

MATERIALS AND METHODS

Study population

Individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai were used in the present study. Certificate of Ethical clearance for the study was obtained from the Institutional Review Board of Ragas Dental College. Twenty patients fitting the aim of the study visiting the dental out-patient facility

Saliva samples were obtained from the patients after obtaining their consent. Twenty patients fitting the aim of the study visiting the dental out-patient facility were included in the study. They were split into two groups of ten each. The first group consisted of periodontally healthy individuals and was designated as **Group H** (Control Group)**.** The second group consisted of individuals who had endosseous implants placed in their oral cavity 6 months post loading of the prosthesis and they were designated as **Group I (**Test Group).

The **American Academy of Periodontology** (AAP) has defined periodontal health as "The condition of a patient when there is function without evidence of disease or abnormality" [4].

The diagnostic definition of peri-implant health is based on the following criteria: 1) absence of peri-implant signs of soft tissue inflammation (redness, swelling, profuse bleeding on probing), and 2) the absence of further additional bone loss following initial healing [72].

In general, however, the probing depth associated with peri-implant health should be ≤5.0 mm[109].

Control group comprised of ten subjects with clinically non-inflammed, healthy gingiva having probing pocket depth of (PPD) lesser than or equal to 3mm, with no signs of clinical attachment loss (CAL) and no bleeding on Probing (BOP).

Test group comprised of ten subjects with endosseous implants placed in their oral cavity 6 months post loading of prosthesis, and the clinical signs taken into consideration were healthy peri-implant gingiva having probing pocket depth of (PPD) lesser than or equal to 4mm, with no signs of clinical attachment loss (CAL) and no bleeding on Probing (BOP).

INCLUSION CRITERIA

- Subjects included in this study were expected to exhibit good general health
- Subjects should have met the criteria of periodontal health as described above

EXCLUSION CRITERIA

- Patient with systemic disorders, such as diabetes mellitus or immunological disorders, HIV.
- Patients on drugs that have potential to interfere with the study such as immunosuppressant drugs or steroids.

- Patients with history of tobacco usage.
- Patients under antimicrobial therapy for the past 6 months

SALIVA SAMPLE COLLECTION:-

- Unstimulated whole saliva was collected in sterile salivary sample tubs in the morning and subjects refrained from eating, drinking, smoking or performing any oral hygiene for at least 2 hours prior to the collection following **Navazesh** method of saliva collection.
- Participants were instructed not to consume food or beverages an hour before the sample collection.
- The patients were asked to rinse their mouth and wait at least 10 minutes before providing a specimen
- Clear and comprehensible labeling of containers was done. (**'H'** for healthy samples and **'I'** for implant samples, each healthy and implant sample designated with a number for keeping count of the patient samples that were collected)
- Samples were frozen and stored at -80°C until the sample collection period is completed.
- Salivary Samples were sent for **Next Generation Sequencing** for microbiome analysis to a laboratory after being packaged in containers with geo packs to maintain the temperature

PCR KIT CONTENTS

- 10mM Tris pH 8.5
- AMPure XP beads
- Freshly Prepared 80% Ethanol (EtOH)
- 96-well 0.2 ml PCR plate
- Microseal 'B' seal
- 96-well MIDI plate

PROCEDURE

DNA extraction, 16S rRNA amplification, library construction and sequencing

Genomic DNA was extracted from the ten saliva samples obtained from patients with dental implants placed in their oral cavity 6 months post loading with the Fast DNA kit and the FastPrep24-5G instrument according to manufacturer's recommendations (MP Biomedicals, Santa Ana, CA). Extracted DNA was then purified with silica-based spin filters (FastDNA kit) and the DNA was amplified using the 16S V3 (341F) forward and V4 (805R) reverse primer pairs with added Illumina adapter overhang nucleotide sequences.

The amplicon generation was done using PCR to amplify a template out of a DNA sample using the region-of-interest specific primers with overhang adapters attached. Amplicon synthesis was then performed using thermocycling with 2.5µl of microbial DNA (5ng/ µl), 5µl of amplicon PCR

forward primer (1µM), 5µl of amplicon PCR reverse primer (1µM), and 12.5µl of 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems) at 95°C. Initial denaturation for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

Reactions were cleaned up with Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the protocol specified by the manufacturer. Attachment of dual indices and Illumina sequencing adapters was performed using 5µl of amplicon PCR product DNA, 5µl of Illumina Nextera XT Index 1 Primer (N7xx) from the Nextera XT Index kit, 5 µl of Nextera XT Index 2 Primer (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10µl of PCR-grade water (UltraClean DNA-free PCR water; MO BIO Laboratories, Inc., Carlsbad, CA, USA), with thermocycling at 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes.

Constructed 16S metagenomic libraries were then purified with Agencourt AMPure XP beads and quantified using a fluorometric quantification method that uses dsDNA binding dyes with Quant-iT PicoGreen and the KAPA Library Quantification Kit (KAPABIOSYSTEMS). Library quality control was performed using the Agilent Technologies 2100 Bioanalyzer to ascertain the quality and average size distribution.

Samples were denatured and diluted to a final concentration of 10 pM with a 20 % PhiX (Illumina) control. Sequencing was performed using the

Illumina Miseq System. After samples are loaded, the MiSeq system provides on instrument secondary analysis using the MiSeq Reporter software (MSR). All eight samples were multiplexed and sequenced in a single lane on the MiSeq using 2 × 300 bp paired-end sequencing. Operational taxonomic units (OTUs) were assigned to each sequence using Human Oral Microbiome Database. The Metagenomics workflow classified organisms from the V3 and V4 amplicon using a database of 16S rRNA data, and this classification is based on the Greengenes database. The output of this workflow is a classification of reads at various taxonomic levels: kingdom, phylum, class, order, family, genus, and species. The analysis output is represented using Bar Graphs, Tables and Cluster Pie Charts.

Statistical analysis

Data was analyzed as recommended by studies conducted previously. Data obtained was compiled systematically using the excel spreadsheet provided by Microsoft Office, the dataset was then subdivided and distributed meaningfully and presented as graphs and tables.

Statistical analysis was performed using the Statistical Package of Social Sciences software (IBM Corp. Released 2011. SPSS Statistics for Windows, version 20.0 Armonk, NY: IBM Corp). Normality test was done using Kolmogorov-Smirnov test and Shapiro-Wilk numerical test, and it was observed that all the variables were normally distributed. Depending on the nature of the data, appropriate parametric statistical tests were chosen. P value of <0.05 was considered to be significant.

Photographs

IMAGE 1: HEALTHY PERIODONTIUM

IMAGE 2: IMPLANT PLACEMENT AND POST LOADING

IMAGE 3: STERILE SALIVARY TUB

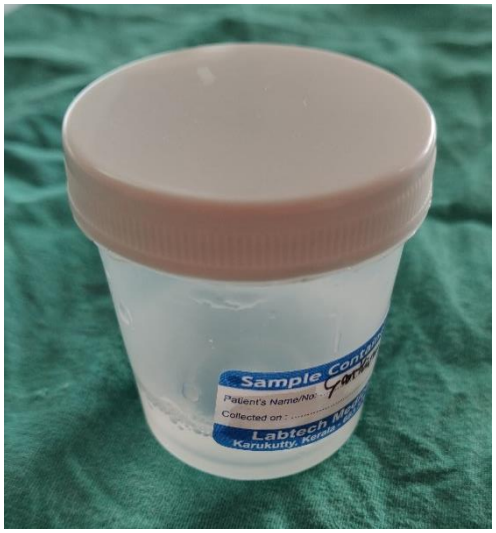


IMAGE 4: ILLUMINA SEQUENCING

IMAGE 5: 4 BASIC STEPS IN ILLUMINA NGS WORK FLOW

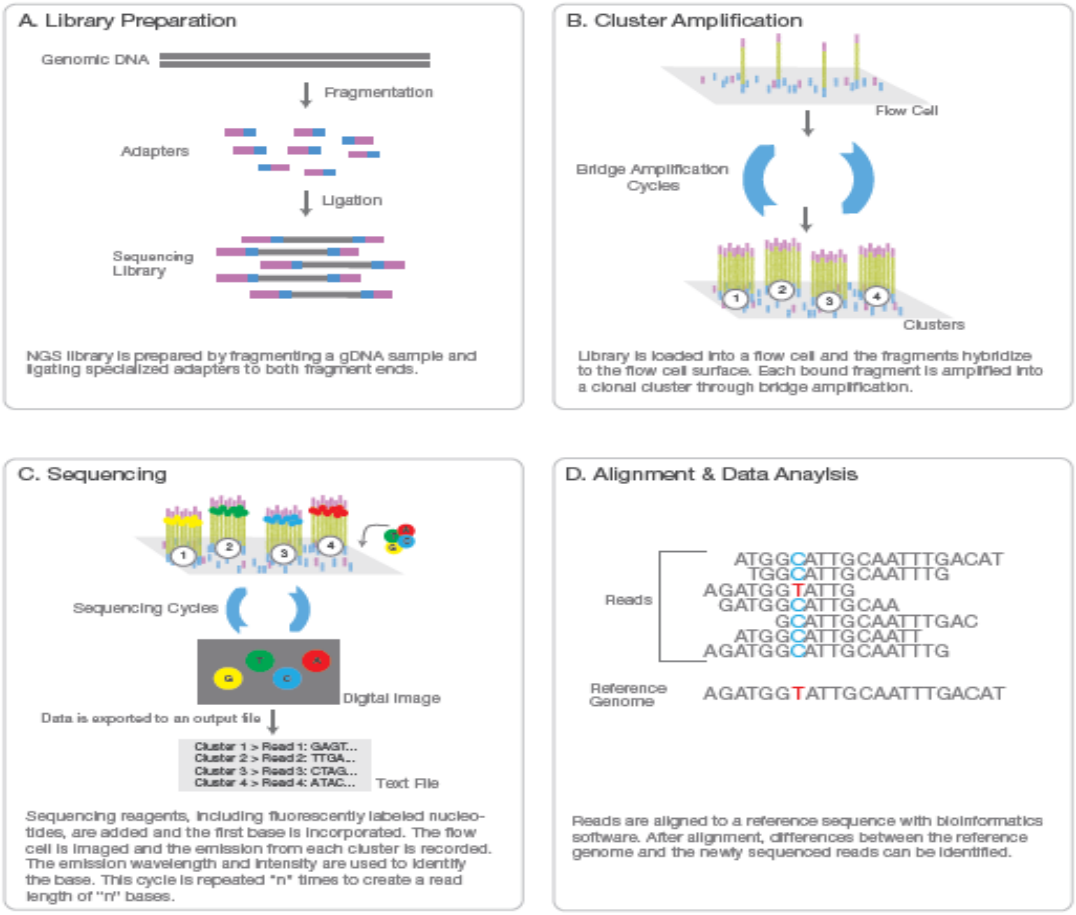
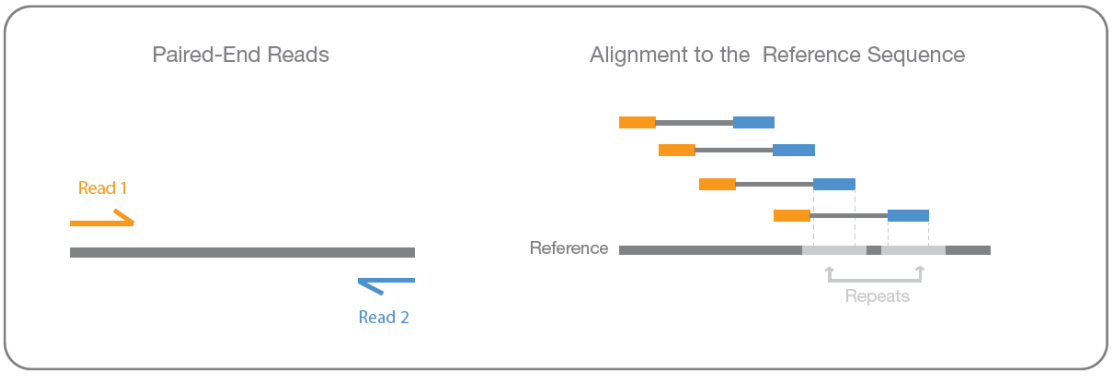
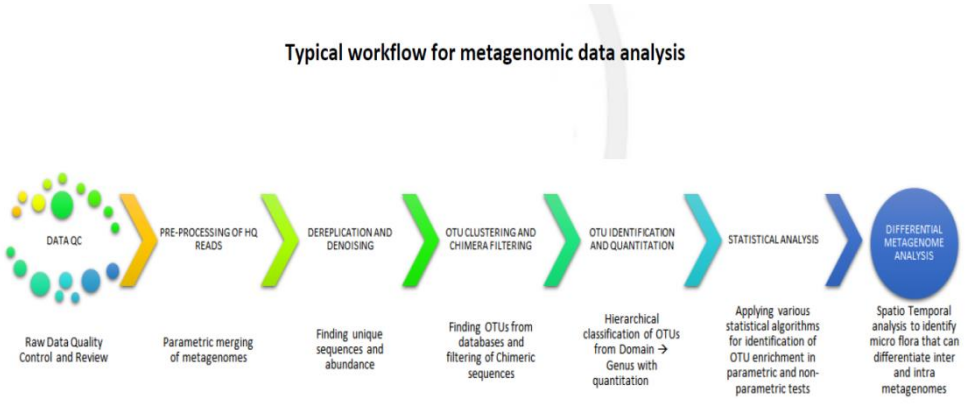


IMAGE 6: PARTIAL END SEQUENCING AND ALIGNMENT





Results

RESULTS

The present study was carried out among a population of twenty individuals seeking dental treatment in Ragas Dental College and Hospital, Chennai. The age of the patients were categorized to be greater than 30 years years with a mean age of 35.4 years.

Unstimulated saliva samples were collected using sterile containers from ten periodontally healthy control individuals (designated as H1, H2, H3 …H10) and from implant patients six months post loading (designated as I1, I2, I3…I10).

Amplicons from V3-V4 hypervariable regions of 16S rRNA gene underwent sequencing. The results obtained from the sequencing process are represented according to taxonomic classification system of bacteria, and the individual comparisons are depicted via tables and graphs.

Distribution of bacteria in healthy and implant sites:

The distribution of bacteria in the healthy and implant sites based on phylum level, genus and species are depicted in Table 1 and Graph 1-A, 1-B and 1-C. In the healthy sites, a total of 10 phyla, 53 genera and 187 species; and in implant sites 11 phyla, 60 genera and 228 species have been identified.

Top 5 phyla in healthy and implant sites:

The top 5 phyla in healthy and implant sites along with their respective abundances are depicted in Table 2 and Graph 2-A and 2-B. In healthy sites, phylum Firmicutes has shown highest abundance of 28.63% followed by *Bacteroidetes* (28.43%), *Fusobacteria* (15.9%), *Proteobacteria* (13.32%) and *Actinobacter* (6.56%). In implant sites, phylum *Firmicutes* (31.7%) has shown the highest abundance of 31.7% followed by *Bacteroidetes* (26.1%), *Fusobacteria* (15.49%), *Proteobacteria* (9.23 %) and TM7 (7.33%). **The phylum not present in healthy samples that was present in implant patients was found to be** *Synergistetes.*

Top 5 genera in healthy and implant sites:

The top 5 genera in healthy and implant sites along with their respective abundances have been depicted in Table 3 and Graph 3-A and 3-B. In both healthy and implant sites, *Prevotella* is the most abundant genus to be identified with an abundance of 12.82% and 12.4% respectively. Following this is *Leptotrichia* with values of 9.14% and 9.23% respectively, the next four genera in healthy sites are *Fusobacterium* (6.75%), *Veillonella* (6.26%), *Porphyromonas* (5.16%) and *Nisseria* (4.97%) and in implant sites are *Saccharibacteria* (6.61%), *Fusobacterium* (6.15%), *Porphyromonas* (5.52%) and *Veillonella* (5.52%). **The genera found in healthy patients that were not present in implant patients are:** *Adlercreutzia, Anaerococcus,*

Bergeyella, Clostridiales, Johnsonella, Odoribacter, Johnsonella, Pyramidobacter, Scardovia, Sporanaerobacter. **The genera found in patient with implants that were not present in healthy patients are:** *Absconditabacteria, Anaerovorax, Eggerthella, Erwinia, Novosphingobium, Slackia*

Species present in healthy samples that are not present in implant samples:

- *Acinetobacter johnsonii*
- *Actinomyces sp. HMT 175*
- *Aggregatibacter paraphrophilus*
- *Aggregatibacter sp. HMT 513*
- *Bifidobacterium animalis*
- *Capnocytophaga sp. HMT 878*
- *Eggerthella lenta*
- *Enterobacter cancerogenus*
- *Enterococcus casseliflavus*
- *Enterococcus saccharolyticus*
- *Filifactor alocis*
- *Gemella bergeri*
- *Haemophilus influenzae*
- *Kingella sp. HMT 932*
- *Klebsiella aerogenes*
- *Lactobacillus crispatus*
- *Novosphingobium panipatense*
- *Peptostreptococcaceae [XI][G-5] bacterium HMT 493*
- *Prevotella sp. HMT 304*
- *Slackia exigua*

Species present in implant samples that are not present in healthy samples:

- *Actinomyces oris*
- *Actinomyces sp. HMT 169*
- *Anaerococcus octavius*
- *Bacteroides heparinolyticus*
- *Bacteroidetes [G-3] bacterium HMT 280*
- *Bacteroidetes [G-3] bacterium HMT 436*
- *Bacteroidetes [G-5] bacterium HMT 505*
- *Bacteroidetes [G-6] bacterium HMT 516*
- *Bulleidia extructa*
- *Butyrivibrio sp. HMT 080*
- *Capnocytophaga sp. HMT 324*
- *Capnocytophaga sp. HMT 338*
- *Clostridiales [F-1][G-1] bacterium HMT 093*
- *Dialister pneumosintes*
- *Enterococcus faecalis*
- *Fretibacterium fastidiosum*
- *Fretibacterium sp. HMT 360*
- *Gracilibacteria (GN02) [G-1] bacterium HMT 871*
- *Johnsonella sp. HMT 166*
- *Lactobacillus fermentum*
- *Lactobacillus gasseri*
- *Lactobacillus salivarius*
- *Leptotrichia sp. HMT 879*
- *Neisseria sp. HMT 018*
- *Oribacterium sp. HMT 102*
- *Peptoniphilaceae [G-2] bacterium HMT 790*
- *Peptostreptococcaceae [XI][G-1] infirmum*
- *Peptostreptococcaceae [XI][G-4] bacterium HMT 369*
- *Peptostreptococcaceae [XI][G-5] saphenum*
- *Peptostreptococcaceae [XI][G-6] minutum*
- *Peptostreptococcaceae [XI][G-6] nodatum*
- *Peptostreptococcaceae [XI][G-7] bacterium HMT 081*
- *Prevotella dentalis*
- *Prevotella enoeca*
- *Prevotella multiformis*
- *Prevotella multisaccharivorax*
- *Prevotella sp. HMT 526*

- *Pseudopropionibacterium propionicum*
- *Pyramidobacter piscolens*
- *Saccharibacteria (TM7) [G-1] bacterium HMT 488*
- *Scardovia wiggsiae*
- *Selenomonas artemidis*
- *Selenomonas dianae*
- *Selenomonas sp. HMT 126*
- *Selenomonas sp. HMT 133*
- *Selenomonas sp. HMT 137*
- *Selenomonas sp. HMT 146*
- *Selenomonas sp. HMT 149*
- *Selenomonas sp. HMT 388*
- *Selenomonas sp. HMT 442*
- *Selenomonas sp. HMT 479*
- *Stomatobaculum sp. HMT 097*
- *Streptococcus sobrinus*
- *Treponema amylovorum*
- *Treponema parvum*
- *Treponema sp. HMT 230*
- *Treponema sp. HMT 231*
- *Treponema sp. HMT 238*
- *Treponema sp. HMT 249*
- *Treponema sp. HMT 258*
- *Treponema sp. HMT 517*
- *Treponema vincentii*
- *Veillonella sp. HMT 917*
- *Veillonellaceae [G-1] bacterium HMT 135*
- *Veillonellaceae [G-1] bacterium HMT 145*
- *Veillonellaceae [G-1] bacterium HMT 483*

Comparison of subgingival microbiome in healthy versus gingival recession sites at genus level: phylogenetic tree

The subgingival microbiome was compared between healthy and gingival recession sites at genus level and is represented in the form of a circular phylogenetic tree in Graph 3-C. The tree has been constructed with phyloT software and is displayed using iTOL (**Letunic and Bork, 2011**). The

bars on the outer band (maroon), represents the abundance of bacterial genera in healthy (green) and implant sites (blue) sites.

TABLE 1: DISTRIBUTION OF BACTERIA IN HEALTHY AND IMPLANT

CRITERIA	PHYLUM	GENUS	SPECIES
HEALTH	10	53	187
IMPLANT	11	60	228

TABLE 2: TOP 5 PHYLA IN HEALTH AND IMPLANT

S.No	HEALTH	ABUNDANCE (%)	IMPLANT	ABUNDANCE (%)
1	Firmicutes	28.62823	Firmicutes	31.7029
2	Bacteroidetes	28.42942	Bacteroidetes	26.08696
3	Fusobacteria	15.90457	Fusobacteria	15.48913
4	Proteobacteria	13.32008	Proteobacteria	9.23913
5	Actinobacteria	6.560636	TM7	7.336957

TABLE 3: TOP 20 GENERA IN HEALTH AND IMPLANT

S.No	HEALTH	ABUNDANCE (%)	IMPLANT	ABUNDANCE (%)
1	Prevotella	12.82306	Prevotella	12.40942
2	Leptotrichia	9.145129	Leptotrichia	9.32971

3	Fusobacterium	6.759443	Saccharibacteria	6.612319
4	Veillonella	6.262425	Fusobacterium	6.15942
5	Porphyromonas	5.168986	Porphyromonas	5.525362
6	Neisseria	4.970179	Veillonella	5.525362
7	Streptococcus	4.970179	Streptococcus	5.344203
8	Saccharibacteria	4.771372	Capnocytophaga	4.891304
9	Capnocytophaga	4.572565	Selenomonas	2.717391
10	[Prevotella]	3.777336	Oribacterium	2.626812
11	Gemella	2.982107	Gemella	2.536232
12	Oribacterium	2.584493	Neisseria	2.445652
13	Rothia	2.485089	Treponema	1.992754
14	Haemophilus	2.286282	Haemophilus	1.902174
15	Bifidobacterium	2.186879	Campylobacter	1.811594
16	Campylobacter	2.087475	Rothia	1.630435
17	Granulicatella	1.789264	Absconditabacteria	1.630435
18	Absconditabacteria	1.39165	Bifidobacterium	1.539855
19	Actinobacillus	1.39165	Ruminococcus	1.539855
20	Ruminococcaceae	1.39165	Granulicatella	1.358696

TABLE 4: TOP 20 SPECIES IN HEALTH AND IMPLANT

S.No	IMPLANT	ABUNDANCE (%)	HEALTH	ABUNDANCE (%)
1	Uncultered	3.26087	Porphyromonas pasteri	2.683897
2	Porphyromonas pasteri	2.807971	Uncultured	2.683897
3	Fusobacterium nucleatum subsp. vincentii	1.992754	Fusobacterium nucleatum subsp. vincentii	2.186879
4	Capnocytophaga sputigena	1.358696	Veillonella parvula	1.590457
5	Veillonella parvula	1.358696	Capnocytophaga sputigena	1.491054
6	Capnocytophaga leadbetteri	1.177536	Alloprevotella sp. HMT 473	1.192843
7	Capnocytophaga granulosa	1.086957	Prevotella veroralis	1.192843
8	Megasphaera micronuciformis	0.996377	Fusobacterium periodonticum	1.093439
9	Catonella morbi	0.905797	Bergeyella sp. HMT 322	0.994036
10	Fusobacterium periodonticum	0.905797	Campylobacter concisus	0.994036
11	Gemella morbillorum	0.905797	Fusobacterium sp. HMT 370	0.994036
12	Granulicatella adiacens	0.905797	Gemella haemolysans	0.994036

13	Haemophilus parainfluenzae	0.905797	Gemella morbillorum	0.994036
14	Leptotrichia hofstadii	0.905797	Gemella sanguinis	0.994036
15	Oribacterium parvum	0.905797	Granulicatella adiacens	0.994036
16	Prevotella sp. HMT 313	0.905797	Haemophilus parainfluenzae	0.994036
17	Prevotella veroralis	0.905797	Neisseria flava	0.994036
18	Saccharibacteria (TM7) [G-1] bacterium HMT 352	0.905797	Oribacterium parvum	0.994036
19	Saccharibacteria (TM7) [G-3] bacterium HMT 351	0.905797	Oribacterium sinus	0.994036
20	Saccharibacteria (TM7) [G-6] bacterium HMT 870	0.905797	Prevotella melaninogenica	0.994036

TABLE 5: PERIODONTAL HEALTH-ASSOCIATED MICROBIOME

(187 species- present in health, not in implant)

	A	93	Neisseria flava
1	Abiotrophia defectiva	94	Neisseria flavescens
2	Absconditabacteria (SR1) [G-1] bacterium HMT 345	95	Neisseria oralis
3	Absconditabacteria (SR1) [G-1] bacterium HMT 874	96	Neisseria perflava
4	Absconditabacteria (SR1) [G-1] bacterium HMT 875	97	Neisseria polysaccharea
5	Actinomyces graevenitzii	98	Neisseria subflava
6	Actinomyces sp. HMT 172		O
7	Actinomyces sp. HMT 180	99	Oribacterium asaccharolyticum
8	Aggregatibacter aphrophilus	100	Oribacterium parvum
9	Aggregatibacter paraphrophilus	101	Oribacterium sinus
10	Aggregatibacter segnis		P
11	Aggregatibacter sp. HMT 458	102	Parvimonas micra
12	Alloprevotella rava	103	Parvimonas sp. HMT 393
13	Alloprevotella sp. HMT 308	104	Peptostreptococcaceae [XI][G-1] sulci
14	Alloprevotella sp. HMT 473	105	Peptostreptococcaceae [XI][G-5] bacterium HMT 493
15	Alloprevotella sp. HMT 914	106	Peptostreptococcaceae [XI][G-7] bacterium HMT 922
16	Alloprevotella tannerae	107	Peptostreptococcaceae [XI][G-7] yurii subspp. yurii & margaretiae
17	Atopobium parvulum	108	Peptostreptococcaceae [XI][G-9] brachy
18	Atopobium rimae	109	Peptostreptococcus stomatis
	B	110	Porphyromonas catoniae
19	Bacteroidales [G-2] bacterium HMT 274	111	Porphyromonas endodontalis
20	Bergeyella sp. HMT 206	112	Porphyromonas gingivalis
21	Bergeyella sp. HMT 322	113	Porphyromonas pasteri

22	Bifidobacterium breve	114	Porphyromonas sp. HMT 275
23	Bifidobacterium dentium	115	Porphyromonas sp. HMT 277
24	Bifidobacterium longum	116	Porphyromonas sp. HMT 278
25	Bifidobacterium scardovii	117	Porphyromonas sp. HMT 284
	C	118	Porphyromonas sp. HMT 285
26	Campylobacter concisus	119	Porphyromonas sp. HMT 930
27	Campylobacter gracilis	120	Prevotella aurantiaca
28	Campylobacter rectus	121	Prevotella denticola
29	Campylobacter sp. HMT 044	122	Prevotella histicola
30	Capnocytophaga granulosa	123	Prevotella loescheii
31	Capnocytophaga leadbetteri	124	Prevotella maculosa
32	Capnocytophaga sp. HMT 326	125	Prevotella melaninogenica
33	Capnocytophaga sp. HMT 332	126	Prevotella micans
34	Capnocytophaga sp. HMT 335	127	Prevotella nanceiensis
35	Capnocytophaga sp. HMT 336	128	Prevotella nigrescens
36	Capnocytophaga sputigena	129	Prevotella oralis
37	Cardiobacterium hominis	130	Prevotella oris
38	Catonella morbi	131	Prevotella oulorum
39	Corynebacterium durum	132	Prevotella pallens
	D	133	Prevotella pleuritidis
40	Dialister invisus	134	Prevotella salivae
	E	135	Prevotella scopos
41	Eggerthella lenta	136	Prevotella shahii
42	Enterococcus durans	137	Prevotella sp. HMT 305
43	Enterococcus italicus	138	Prevotella sp. HMT 306
44	Escherichia coli	139	Prevotella sp. HMT 309
	F	140	Prevotella sp. HMT 313

45	Finegoldia magna	141	Prevotella sp. HMT 314
46	Fusobacterium naviforme	142	Prevotella sp. HMT 317
47	Fusobacterium nucleatum subsp. animalis	143	Prevotella sp. HMT 443
48	Fusobacterium nucleatum subsp. polymorphum	144	Prevotella sp. HMT 472
49	Fusobacterium nucleatum subsp. vincentii	145	Prevotella sp. HMT 475
50	Fusobacterium periodonticum	146	Prevotella veroralis
51	Fusobacterium sp. HMT 203		R
52	Fusobacterium sp. HMT 370	147	Rothia aeria
	G	148	Rothia dentocariosa
53	Gemella haemolysans	149	Rothia mucilaginosa
54	Gemella morbillorum	150	Ruminococcaceae [G-1] bacterium HMT 075
55	Gemella sanguinis	151	Ruminococcaceae [G-2] bacterium HMT 085
56	Gracilibacteria (GN02) [G-2] bacterium HMT 873		S
57	Granulicatella adiacens	152	Saccharibacteria (TM7) [G-1] bacterium HMT 346
58	Granulicatella elegans	153	Saccharibacteria (TM7) [G-1] bacterium HMT 347
	H	154	Saccharibacteria (TM7) [G-1] bacterium HMT 348
59	Haemophilus haemolyticus	155	Saccharibacteria (TM7) [G-1] bacterium HMT 349
60	Haemophilus parainfluenzae	156	Saccharibacteria (TM7) [G-1] bacterium HMT 352
61	Haemophilus paraphrohaemolyticus	157	Saccharibacteria (TM7) [G-1] bacterium HMT 869
62	Haemophilus pittmaniae	158	Saccharibacteria (TM7) [G-2] bacterium HMT 350
63	Haemophilus sp. HMT 036	159	Saccharibacteria (TM7) [G-3] bacterium HMT 351
64	Haemophilus sp. HMT 908	160	Saccharibacteria (TM7) [G-5] bacterium HMT 356
65	Haemophilus sputorum	161	Saccharibacteria (TM7) [G-6] bacterium HMT 870
	K	162	Selenomonas noxia

66	Kingella oralis	163	Selenomonas sp. HMT 136
67	Klebsiella pneumoniae	164	Shuttleworthia satelles
	L	165	Solobacterium moorei
68	Lachnoanaerobaculum orale	166	Stomatobaculum longum
69	Lachnoanaerobaculum umeaense	167	Stomatobaculum sp. HMT 097
70	Lautropia mirabilis	168	Streptococcus lactarius
71	Leptotrichia buccalis	169	Streptococcus mitis
72	Leptotrichia goodfellowii	170	Streptococcus oralis subsp. dentisani clade 058
73	Leptotrichia hofstadii	171	Streptococcus parasanguinis clade 411
74	Leptotrichia hongkongensis	172	Streptococcus peroris
75	Leptotrichia shahii	173	Streptococcus pneumoniae
76	Leptotrichia sp. HMT 212	174	Streptococcus salivarius
77	Leptotrichia sp. HMT 215	175	Streptococcus sanguinis
78	Leptotrichia sp. HMT 217	176	Streptococcus thermophilus
79	Leptotrichia sp. HMT 218		T
80	Leptotrichia sp. HMT 221	177	Tannerella forsythia
81	Leptotrichia sp. HMT 225	178	Tannerella sp. HMT 286
82	Leptotrichia sp. HMT 392	179	Tannerella sp. HMT 808
83	Leptotrichia sp. HMT 417	180	Treponema socranskii
84	Leptotrichia sp. HMT 463		U
85	Leptotrichia sp. HMT 498	181	Uncultured
86	Leptotrichia sp. HMT 847		V
87	Leptotrichia wadei	182	Veillonella atypica
	M	183	Veillonella denticariosi
88	Megasphaera micronuciformis	184	Veillonella dispar
89	Mycoplasma orale	185	Veillonella parvula
90	Mycoplasma salivarium	186	Veillonella rogosae

	N	187	Veillonella sp. HMT 780
91	Neisseria cinerea		
92	Neisseria elongata		

TABLE 6: IMPLANT ASSOCIATED MICROBIOME (228 species)

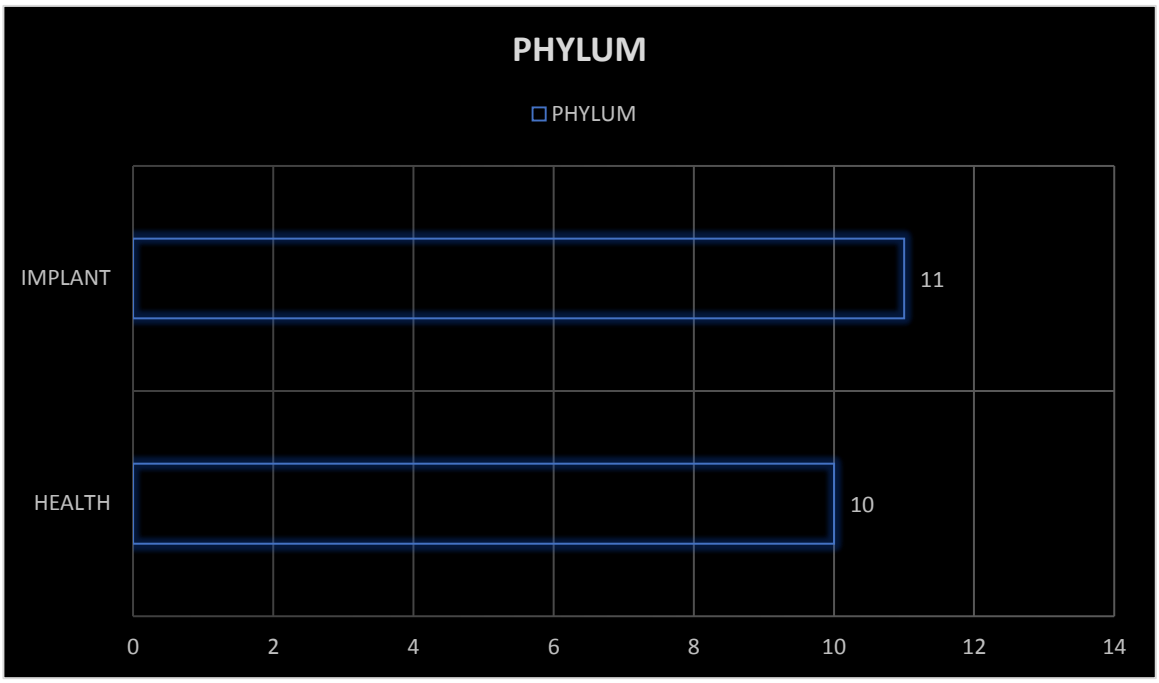
	A	115	Oribacterium parvum
1	Abiotrophia defectiva	116	Oribacterium sinus
2	Absconditabacteria (SR1) [G-1] bacterium HMT 345	117	Oribacterium sp. HMT 078
3	Absconditabacteria (SR1) [G-1] bacterium HMT 874		P
4	Absconditabacteria (SR1) [G-1] bacterium HMT 875	118	Parvimonas micra
5	Actinomyces graevenitzii	119	Parvimonas sp. HMT 393
6	Actinomyces sp. HMT 180	120	Peptostreptococcaceae [XI][G-1] sulci
7	Aggregatibacter segnis	121	Peptostreptococcaceae [XI][G-7] bacterium HMT 081
8	Aggregatibacter sp. HMT 458	122	Peptostreptococcaceae [XI][G-9] brachy
9	Aggregatibacter sp. HMT 949	123	Peptostreptococcus stomatis
10	Alloprevotella rava	124	Porphyromonas catoniae
11	Alloprevotella sp. HMT 308	125	Porphyromonas endodontalis
12	Alloprevotella sp. HMT 473	126	Porphyromonas gingivalis
13	Alloprevotella tannerae	127	Porphyromonas pasteri
14	Anaeroglobus geminatus	128	Porphyromonas sp. HMT 275
15	Atopobium parvulum	129	Porphyromonas sp. HMT 277
16	Atopobium rimae	130	Porphyromonas sp. HMT 278
	B	131	Porphyromonas sp. HMT 284
17	Bacteroidaceae [G-1] bacterium HMT 272	132	Porphyromonas sp. HMT 285
18	Bacteroidales [G-2] bacterium HMT 274	133	Porphyromonas sp. HMT 930
19	Bacteroides heparinolyticus	134	Prevotella aurantiaca
20	Bacteroidetes [G-3] bacterium HMT 280	135	Prevotella baroniae
21	Bacteroidetes [G-3] bacterium HMT 436	136	Prevotella buccae
22	Bacteroidetes [G-5] bacterium HMT 511	137	Prevotella denticola
23	Bacteroidetes [G-6] bacterium HMT 516	138	Prevotella histicola
24	Bergeyella sp. HMT 206	139	Prevotella intermedia
25	Bergeyella sp. HMT 322	140	Prevotella loescheii
26	Bifidobacterium breve	141	Prevotella melaninogenica

27	Bifidobacterium dentium	142	Prevotella nanceiensis
28	Bifidobacterium longum	143	Prevotella nigrescens
29	Bifidobacterium scardovii	144	Prevotella oris
30	Butyrivibrio sp. HMT 080	145	Prevotella oulorum
31	Butyrivibrio sp. HMT 455	146	Prevotella pallens
	C	147	Prevotella pleuritidis
32	Campylobacter concisus	148	Prevotella saccharolytica
33	Campylobacter gracilis	149	Prevotella salivae
34	Campylobacter rectus	150	Prevotella scopos
35	Campylobacter sp. HMT 044	151	Prevotella shahii
36	Capnocytophaga granulosa	152	Prevotella sp. HMT 300
37	Capnocytophaga leadbetteri	153	Prevotella sp. HMT 301
38	Capnocytophaga sp. HMT 323	154	Prevotella sp. HMT 306
39	Capnocytophaga sp. HMT 326	155	Prevotella sp. HMT 309
40	Capnocytophaga sp. HMT 332	156	Prevotella sp. HMT 313
41	Capnocytophaga sp. HMT 336	157	Prevotella sp. HMT 314
42	Capnocytophaga sp. HMT 412	158	Prevotella sp. HMT 317
43	Capnocytophaga sp. HMT 863	159	Prevotella sp. HMT 443
44	Capnocytophaga sp. HMT 864	160	Prevotella sp. HMT 472
45	Capnocytophaga sputigena	161	Prevotella sp. HMT 526
46	Cardiobacterium hominis	162	Prevotella veroralis
47	Catonella morbi		R
48	Centipeda periodontii	163	Rothia aeria
49	Corynebacterium durum	164	Rothia dentocariosa
	D	165	Rothia mucilaginosa
50	Dialister invisus	166	Ruminococcus [G-1] bacterium HMT 075
	E	167	Ruminococcus [G-2] bacterium HMT 085
51	Eikenella corrodens		S
	F	168	Saccharibacteria (TM7) [G-1] bacterium HMT 346
52	Filifactor alocis	169	Saccharibacteria (TM7) [G-1] bacterium HMT 347
53	Finegoldia magna	170	Saccharibacteria (TM7) [G-1] bacterium HMT 348
54	Fretibacterium sp. HMT 362	171	Saccharibacteria (TM7) [G-1] bacterium HMT 349
55	Fusobacterium naviforme	172	Saccharibacteria (TM7) [G-1] bacterium HMT 352
56	Fusobacterium nucleatum subsp. animalis	173	Saccharibacteria (TM7) [G-1] bacterium HMT 488

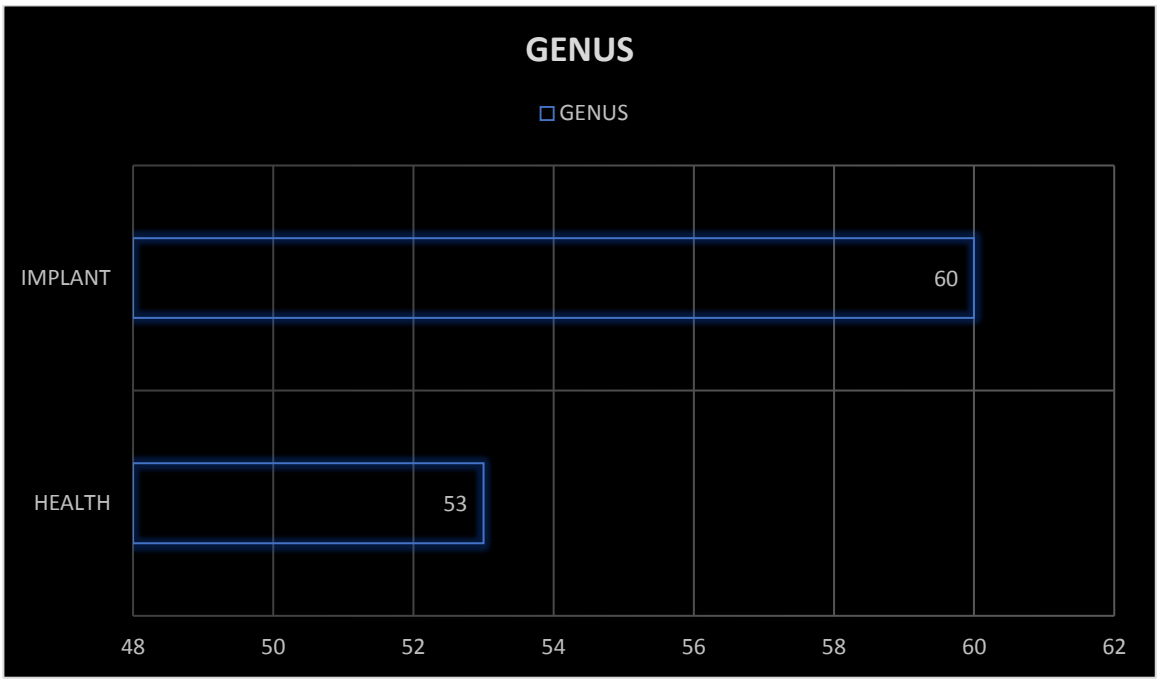
57	Fusobacterium nucleatum subsp. nucleatum	174	Saccharibacteria (TM7) [G-2] bacterium HMT 350
58	Fusobacterium nucleatum subsp. polymorphum	175	Saccharibacteria (TM7) [G-3] bacterium HMT 351
59	Fusobacterium nucleatum subsp. vincentii	176	Saccharibacteria (TM7) [G-5] bacterium HMT 356
60	Fusobacterium periodonticum	177	Saccharibacteria (TM7) [G-6] bacterium HMT 870
61	Fusobacterium sp. HMT 203	178	Saccharibacteria (TM7) [G-8] bacterium HMT 955
62	Fusobacterium sp. HMT 370	179	Selenomonas dianae
	G	180	Selenomonas noxia
63	Gemella haemolysans	181	Selenomonas sp. HMT 134
64	Gemella morbillorum	182	Selenomonas sp. HMT 136
65	Gemella sanguinis	183	Selenomonas sp. HMT 137
66	Gracilibacteria (GN02) [G-1] bacterium HMT 871	184	Selenomonas sp. HMT 146
67	Gracilibacteria (GN02) [G-1] bacterium HMT 872	185	Selenomonas sp. HMT 478
68	Gracilibacteria (GN02) [G-2] bacterium HMT 873	186	Selenomonas sp. HMT 479
69	Granulicatella adiacens	187	Selenomonas sputigena
70	Granulicatella elegans	188	Shuttleworthia satelles
	H	189	Solobacterium moorei
71	Haemophilus haemolyticus	190	Stomatobaculum longum
72	Haemophilus parainfluenzae	191	Stomatobaculum sp. HMT 097
73	Haemophilus paraphrohaemolyticus	192	Streptococcus anginosus
74	Haemophilus pittmaniae	193	Streptococcus cristatus clade 578
75	Haemophilus sp. HMT 036	194	Streptococcus gordonii
76	Haemophilus sp. HMT 908	195	Streptococcus lactarius
77	Haemophilus sputorum	196	Streptococcus mitis
	J	197	Streptococcus parasanguinis clade 411
78	Johnsonella sp. HMT 166	198	Streptococcus peroris
	K	199	Streptococcus pneumoniae
79	Kingella denitrificans	200	Streptococcus salivarius
80	Kingella oralis	201	Streptococcus sanguinis
81	Kingella sp. HMT 012	202	Streptococcus sinensis
	L	203	Streptococcus thermophilus
82	Lachnoanaerobaculum orale		T
83	Lachnoanaerobaculum umeaense	204	Tannerella forsythia
84	Lachnospiraceae [G-2] bacterium HMT 096	205	Tannerella sp. HMT 286
85	Lactobacillus salivarius	206	Tannerella sp. HMT 808

86	Lautropia mirabilis	207	Treponema amylovorum
87	Leptotrichia buccalis	208	Treponema denticola
88	Leptotrichia hofstadii	209	Treponema lecithinolyticum
89	Leptotrichia hongkongensis	210	Treponema maltophilum
90	Leptotrichia shahii	211	Treponema medium
91	Leptotrichia sp. HMT 212	212	Treponema parvum
92	Leptotrichia sp. HMT 215	213	Treponema socranskii
93	Leptotrichia sp. HMT 217	214	Treponema sp. HMT 231
94	Leptotrichia sp. HMT 218	215	Treponema sp. HMT 237
95	Leptotrichia sp. HMT 219	216	Treponema sp. HMT 249
96	Leptotrichia sp. HMT 221	217	Treponema sp. HMT 258
97	Leptotrichia sp. HMT 225	218	Treponema vincentii
98	Leptotrichia sp. HMT 392		U
99	Leptotrichia sp. HMT 417	219	Uncultured
100	Leptotrichia sp. HMT 463		V
101	Leptotrichia sp. HMT 498	220	Veillonella atypica
104	Leptotrichia wadei	221	Veillonella denticariosi
	M	222	Veillonella dispar
105	Megasphaera micronuciformis	223	Veillonella parvula
106	Mycoplasma faucium	224	Veillonella rogosae
	N	225	Veillonella sp. HMT 780
107	Neisseria cinerea	226	Veillonella sp. HMT 917
108	Neisseria elongata	227	Veillonellaceae [G-1] bacterium HMT 145
109	Neisseria flava	228	Veillonellaceae [G-1] bacterium HMT 155
110	Neisseria flavescens		
	O		
114	Oribacterium asaccharolyticum		

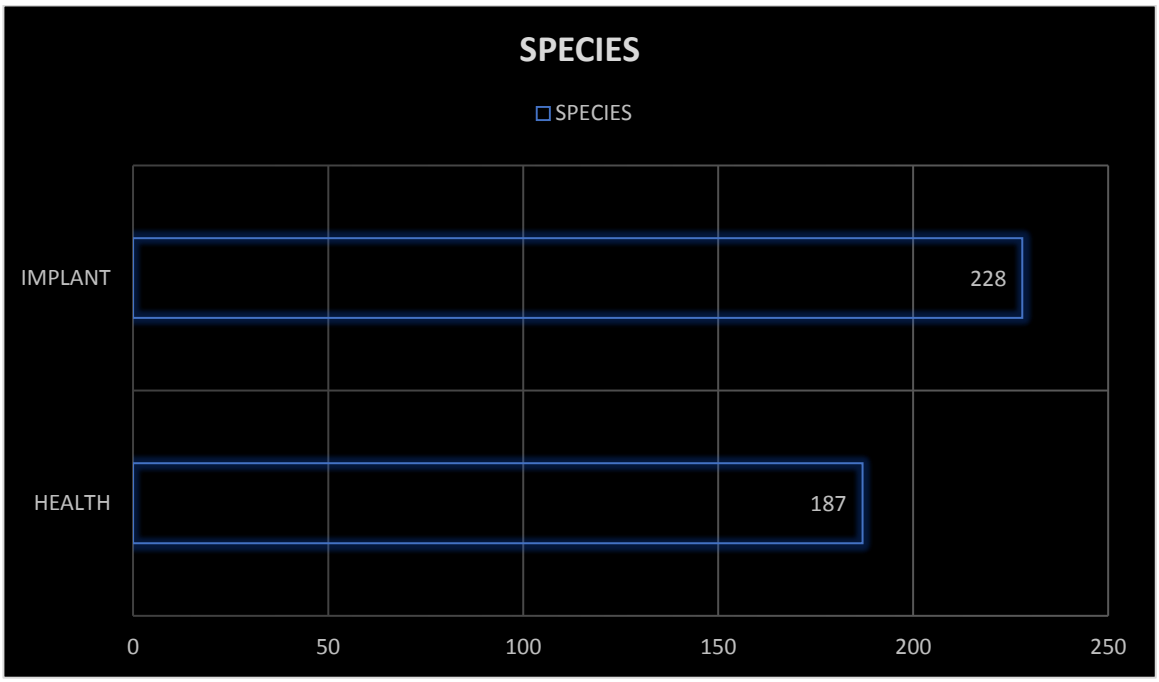
GRAPH 1-A: BAR GRAPH DEMONSTRATING DISTRIBUTION OF BACTERIAL PHYLUM IN HEALTH AND IMPLANT



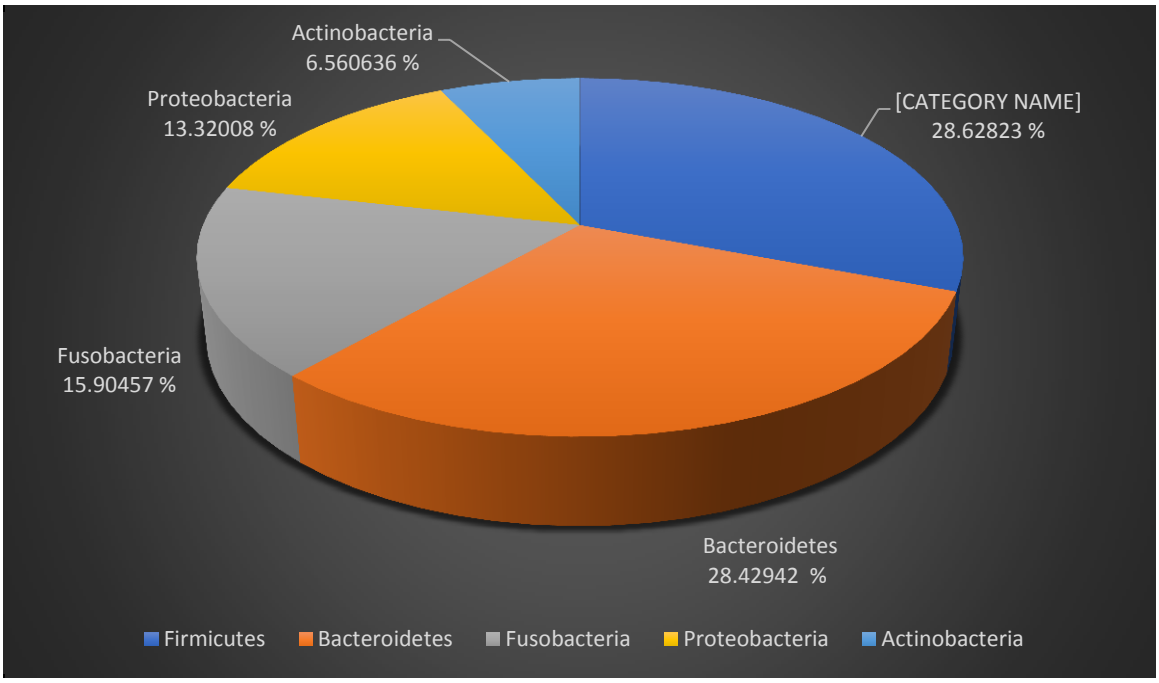
GRAPH 1-B: BAR GRAPH DEMONSTRATING DISTRIBUTION OF BACTERIAL GENUS IN HEALTH AND IMPLANT



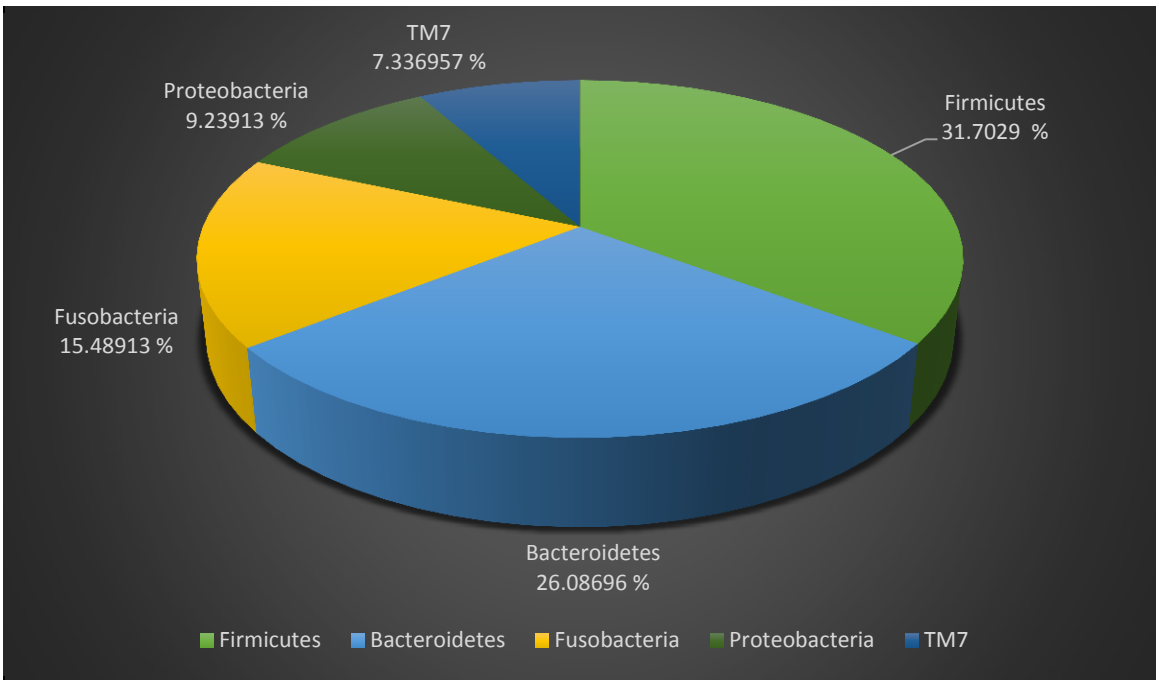
GRAPH 1-C: BAR GRAPH DEMONSTRATING DISTRIBUTION OF BACTERIAL SPECIES IN HEALTH AND IMPLANT



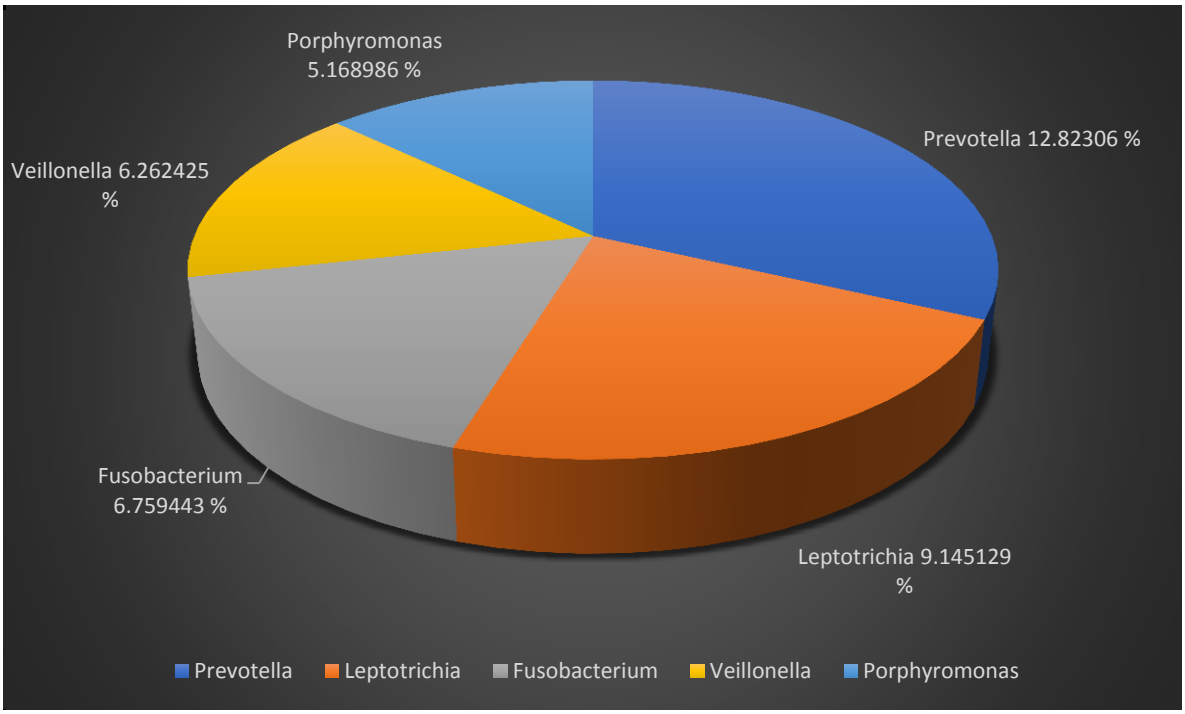
GRAPH 2-A: TOP 5 PHYLUM IN HEALTH



GRAPH 2-B: TOP 5 PHYLA IMPLANT



GRAPH 3-A: TOP 5 GENERA IN HEALTH



GRAPH 3-B: TOP 5 GENERA IN IMPLANT

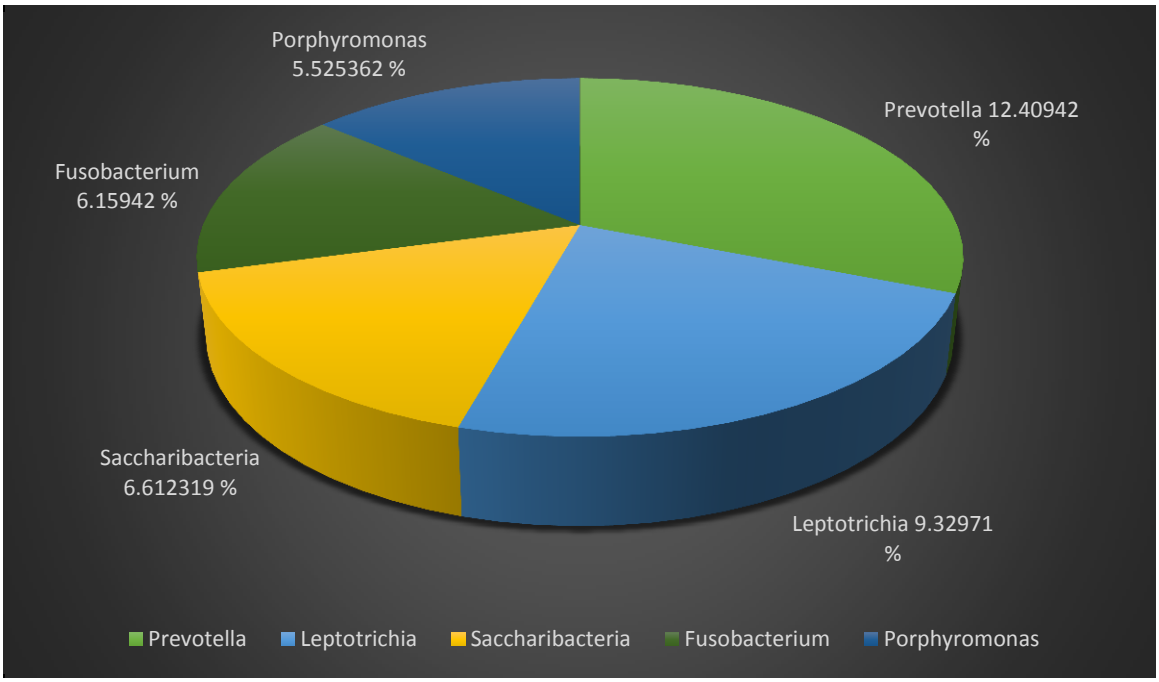
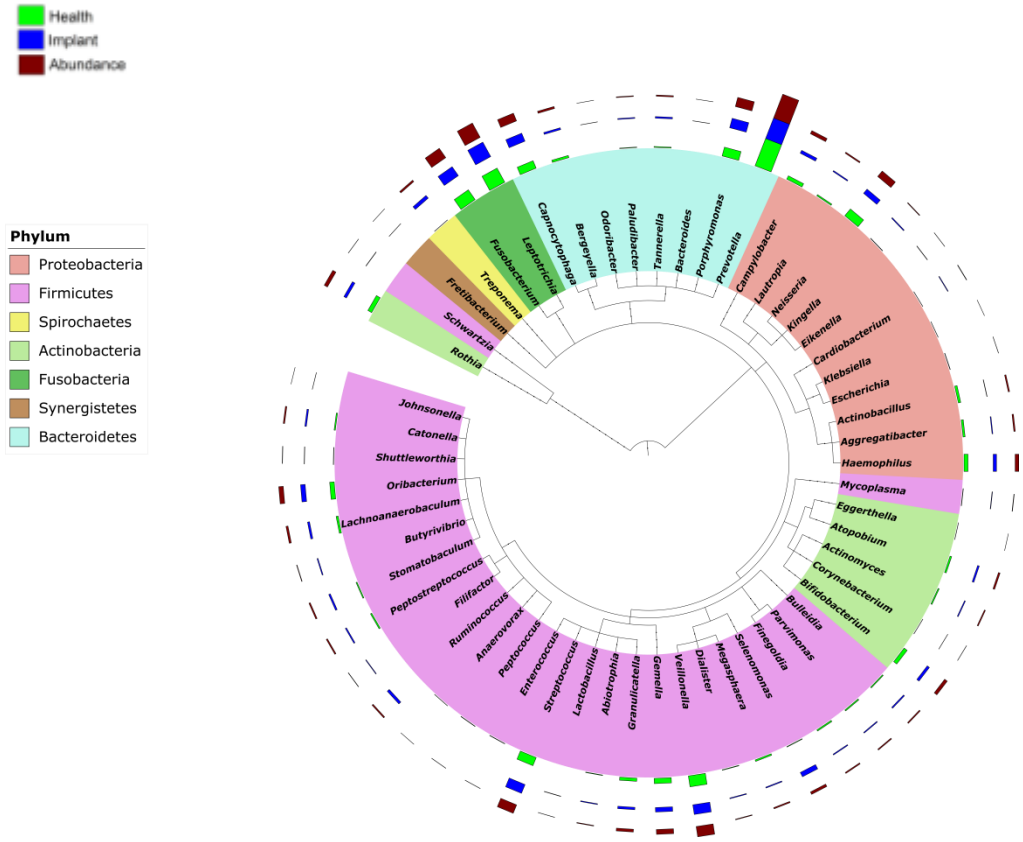


TABLE 3-C: PHYLOGENETIC TREE AT GENUS LEVEL



Discussion

DISCUSSION

The simple presence of oral microbiota in the mouth inhibits colonisation by pathogens, the phenomenon of colonisation resistance. Because all surfaces of the mouth are colonised by commensals there are few binding sites available for pathogens [121].

The human salivary microbiome may be a factor that plays a role in diseases of the oral cavity and interacts with microbiomes from other parts of the human body (in particular, the intestinal tract), but only little is known about normal variation in the salivary microbiome. The main reason for analyzing the human salivary microbiome is that since saliva is increasingly preferred for sampling in humans, as a source of DNA for epidemiologic and population genetic studies [19], it would be useful to identify bacterial taxa in saliva which in turn will be able to provide us with insights into human population structure and migrations.

Bacteria that normally reside in the oral cavity (i.e. the indigenous microbiota) can select from different ecosystems for their habitat. Most pathogenic species (with the exception of spirochetes who limit themselves to the pocket) are able to colonise all five niches of the oral cavity [28].Some periodontopathogens (*F. nucleatum* and *P. intermedia*) are involved in the aetiology of tonsillitis, while others can even colonise the maxillary sinus. Since most pathogens are found in more than one niche, it is reasonable to assume that transmission occurs between these intra-oral niches **(*called***

translocation) occurs. The existence of such a translocation was illustrated by **Quirynen et al. in 1999** [79]. Such a translocation is made possible only in the presence of a fluid medium such as saliva.

An inflammation that is of peri-implant origin usually represents the presence of a disease after the placement of a dental implant, which affects both the surrounding hard and soft tissue. Due to the high prevalence of peri-implantitis, routine check-ups and evaluation along with elimination of risk factors (e.g. smoking, systemic diseases and specific continuous periodontitis**)** are recommended maintenance measures [57]**.** In addition to the different aspects involved in the process of osseointegration, the type and structure of the implant surface are also of importance [27, 65]. For the treatment of peri-implant disease conditions various conservative and surgical approaches can be utilised. This however recognises that treatment of peri-implantitis does not yield the same optimal results in terms of surgical and non-surgical management of periodontal diseases.

The need for biomarker based assessment of peri-implant sites that are likely to undergo destruction is therefore great.

There are hundreds of uncultivated bacterial species still remaining in the human oral cavity, and thus the role of the oral microbiome in health and disease is not yet fully understood. Previous studies which were conducted solely focused on the pathogens of teeth and implant and to explore the influence of implants on the oral microbiome. [2].

A number of in vitro studies have been published documenting the bacterial leakage along the interface at implant-abutment assemblies. Essentially, these indicated a bidirectional flux of fluids and bacteria via the microgap [117], which occurred within days following placement, at all implant types and implant-abutment configurations [98.] The phenomenon was reduced when the closing torque of the abutment screw was increased.

In addition, more bacterial penetration was found along the transversal screw of screw-retained assemblies when compared with the microgap separating the implant from the abutment.

In a study conducted by **Lee KH et al** [58] focused on exploring possible oral sources of species colonizing the oral cavity in patients with newly placed implants by comparing the pre and post implantation microbiota. Most of the microbial species were found on teeth and on the dorsum of the tongue before the implants were actually placed, indicating that there is an impact of the pre-implant microbiota on microbial colonization of dental implants. This impact of the pre-implantation microbiota was also observed in three subjects that were colonized at relatively lower levels both before and after implant placement. The similarity of the bacterial samples obtained from the patients on the same visit from around the teeth and implants in partially dentate subjects indicate that the teeth are a likely source of implant species has previously been described (**Shulman & Shepard 1989, Quirynen & Listgarten 1990, Meffert 1993, Mombelli et al. 1995**) [72]. It may also reflect a

similarity in chronology and quantity of plaque formation following the anti-microbial regimen associated with implant surgery (**Eke et al. 1995)**.

Previous researchers have established that NGS technology is an open-ended technique recognised for its high-throughput genomic analysis approach and its ability to quantify abundance of bacterial species. Its use has been applied in this study for sequencing the microbiome of unstimulated saliva samples from patients with implants placed in their oral cavity and periodontally healthy subjects. Illumina sequencing, being an advent of NGS technology has been utilised in this study as it provides more sequences per run, analyses a larger sample size, has a better assessment of the microbiome diversity, includes more bar-coded time points and samples, generates and sequences short 16S rRNA amplicons to determine even low abundance mtaxa.

The V3-V4 region of 16S rRNA were used in this study because though V4 region provides a full overlap of two reads and reduces the noise in sequencing data thereby preventing OTU inflation but there is only less information contained within V4 region owing to its length (~255 base pairs) so a longer fragment such as the V3 which spans multiple hypervariable regions was also selected and is more suitable for distinguishing all bacterial species to genus level.

Subjects were periodontally evaluated and allocated into two groups as healthy controls (ten subjects) and patients with implants placed in their oral

cavity, six months after the loading of the prostheses (ten subjects). Sterile containers with measurements were used to collect the unstimulated whole saliva samples based on the reliability as observed in earlier literature. In the experimental workflow, all reactions were carried out with water and plastic materials guaranteed as DNA-free to avoid contamination.

The purpose of the study was to understand the salivary microbiome in an implant environment and since only a few studies have been conducted (**Cássio do Nascimento et al**) keeping this aim in mind, the study was taken up by our department.

A vast diversity of salivary microflora and inter-individual variations among all samples were observed in our study. A total of 10 phyla, 53 genera and 187 species were observed in healthy sites; and11 phyla, 60 genera and 228 species were found in the implant sites.

When the salivary microbiome was analysed at phylum level, with the top five phyla in healthy sites being phylum *Firmicutes* with the highest abundance of (28.63%) followed by *Bacteroidetes* (28.43%), *Fusobacteria* (15.9%), *Proteobacteria* (13.32%) and *Actinobacter* (6.56%), the results of our study fall in line with these earlier studies conducted by **Egija Zaura**

Streptococcus, Selenomonas, Pectinatus and Cohnella also belong to the phylum Firmicutes. Our study also reports a higher abundance of Proteobacteria and Fusobacteria. Proteobacteria phylum comprises of species such as Campylobacter, Kingella, Eikenella and Neisseria and Fusobacteria

phylum comprises genera Fusobacterium and Leptotriciae; all of which are known to be early colonisers.

In implant sites, the top five phyla are phylum Firmicutes (31.7%) which has shown the highest abundance of 31.7% followed by Bacteroidetes (26.1%), Fusobacteria (15.49%), Proteobacteria 9.23 % and TM7 (7.33%). In a study conducted by **Cássio do Nascimento et al** which was similar to the our study, it was observed that the most prevalent species found in patients with titanium implants were the Gram-negative bacteria belonging mainly to the phylum *Proteobacteira*, which includes the *Neisseriaceae* and *Campylobacteraceae* families, followed by the phyla *Firmicutes* and *Actinobacteria* and *Bacteroidetes*, this correlates with the results of our study. The only phyla that was absent in healthy samples was *Synergistetes.* Studies were conducted by **Sonia R. Vartoukian** in which *Synergistetes* was cultured to obtain a new genus *Fretibacterium* and the new species *Fretibacterium fastidiosum* in 2009 [120]. The cells are asaccharolytic and major amounts of acetic acid and moderate amounts of propionic acid are produced as end products of metabolism in peptone-yeast extract-glucose broth supplemented with a filtered cell sonicate of *Fusobacterium nucleatum*.

At genus level, *Prevotella* was the predominant genus among both the healthy sites (12.82%) and implant sites (12.4%) this correlates with the study conducted by **Daniel Blestorm**. The genus *Neisseria* and *Eikenella* were found to be more abundant in the study conducted by **Cássio do Nascimento et al.** Following this is *Leptotrichia* with values of 9.14% and 9.23%

respectively, the next four genera in healthy sites are *Fusobacterium* (6.75%), *Veillonella* (6.26%), *Porphyromonas* (5.16%) and *Nisseria* (4.97%) and in implant sites are *Saccharibacteria* (6.61%), *Fusobacterium* (6.15%), *Porphyromonas* (5.52%) and *Veillonella* (5.52%). This correlates with the results from previous studies conducted by **Sonia R. Vartoukian.**

The genera found in healthy samples that were not in implant samples were: *Adlercreutzia, Anaerococcus, Bergeyella, Clostridiales, Johnsonella, Odoribacter, Johnsonella, Pyramidobacter, Scardovia, Sporanaerobacter*. The genera found in patient with implants that were not present in healthy patients are: *Absconditabacteria, Anaerovorax, Eggerthella, Erwinia, Novosphingobium, Slackia.*

A total of 187 species were identified in health and 228 species identified in implants. Among the top 15 species found in both healthy and implant patients, *Porphyromonas pasteri* showed the highest abundance of 2.68% in health and and 2.81% in implant this was followed by *Fusobacterium nucleaum subsp. vincentii* this correlated with the results based on a comparative study conducted between zirconia and titanium implants, in which the titanium implants showed the presence of *Fusobacterium nucleatum* species more predominantly.

At species level, the top 15 species in healthy samples were *Porphyromonas pasteri* (2.68%) *Fusobacterium nucleatum subsp. Vincentii* (2.18%)*,Veillonella parvula* (1.59%) *Capnocytophaga sputigena* (1.49%), *Alloprevotella sp. HMT 473* (1.19%) *Prevotella veroralis* (1.19%),

Fusobacterium periodonticum (1.09%) *Bergeyella sp. HMT 322* (0.99%), *Campylobacter concisus* (0.99%) *Fusobacterium sp. HMT 370* (0.99%), *Gemella haemolysins* (0.99%), *Gemella morbillorum* (0.99%), *Gemella sanguine*(0.99%), *Granulicatella adiacens*(0.99%), *Haemophilus parainfluenzae* (0.99%)

The top 15 bacterial species found in implants related sites were: *Porphyromonas pasteri* (2.8%), *Fusobacterium nucleatum subsp. Vincentii* (1.99%), *Capnocytophaga sputigena* (1.35%) *Veillonella parvula* (1.35%) *Capnocytophaga leadbetteri* (1.17%), *Capnocytophaga granulosa* (1.086%) *Megasphaera micronuciformis* (0.99%), *Catonella morbi* (0.90%) *Fusobacterium periodonticum* (0.90%), *Gemella morbillorum* (0.90%), *Granulicatella adiacens* (0.90%), *Haemophilus parainfluenzae* (0.90%) *Leptotrichia hofstadii* (0.90%), *Oribacterium parvum* (0.90%), *Prevotella sp. HMT 313* (0.90%). These results were in contrast to the results obtained in the st kudy conducted by **Jan Cosyn et al,** in which it was found that the speices with highest abundance in implants was for *Fusobacterium periodonticum* followed by *Fusobacterium nucleatum sp. Nucleatum*.

Species found exclusively in healthy samples: *Acinetobacter johnsonii Actinomyces sp. HMT 175, Aggregatibacter paraphrophilus, Aggregatibacter sp. HMT 513, Bifidobacterium animalis, Capnocytophaga sp HMT 878, Eggerthella lenta, Enterobactercancerogenus, Enterococcus casseliflavus, Enterococcus saccharolyticus, Filifactor alocis, Gemella bergeri,*

Haemophilus influenzae Kingella sp. HMT 932 , Klebsiella aerogenes, Lactobacillus crispatus, Novosphingobium panipatense, Peptostreptococcaceae [XI][G-5] bacterium HMT 493, Prevotella sp. HMT 304, Slackia exigua

Apart from this species found exclusively in implant related samples were: *Actinomyces oris ,Actinomyces sp. HMT 169 ,Anaerococcus octavius, Bacteroides heparinolyticus,Bacteroidetes [G-3] bacterium HMT 280, Bacteroidetes [G-3] bacterium HMT 436, Bacteroidetes [G-5] bacterium HMT 505 , Bacteroidetes [G-6] bacterium HMT 516,Bulleidia extructa, Butyrivibrio sp. HMT 080, Capnocytophaga sp. HMT 324, Capnocytophaga sp. HMT 338, Clostridiales [F-1][G-1] bacterium HMT 093, Dialister pneumosintes, Enterococcus faecalis, Fretibacterium fastidiosum, Fretibacterium sp. HMT 360, etc* as stated in the results.

This in turn led to the conclusion that a distinct health-associated salivary microbiome and implant-associated salivary microbiome were identified.

Based on previous studies conducted by **Cortelli S** [20] it is proven that a previous history of periodontitis may imply a condition of peri-implantitis in the future, thereby establishing a relationship between periodontal disease and peri-implant conditions.

Limitations of the study:

Due to the presence of vast numbers of uncultured species that have not been accounted for by the HOMI database, there are still a lot of microorganisms that we are unaware of and these microorganisms may be one of the key pathogens that are involved in the pathogenesis of disease.

Summary and Conclusion

SUMMARY AND CONCLUSION

This study characterized salivary microbiome in patients with dental endosseous implants placed in their oral cavity (6 months post loading). Ten unstimulated whole salivary samples obtained from patients with implants placed in their oral cavity (6 months post loading) and ten samples from periodontally healthy were collected and microbiome characterization was done using NGS technology using Illumina sequencing.

A wide array of microbes were identified in health and implant belonging to a complex community structure comprising 10 phyla, 53 genus, 187 species in healthy subjects and 11 phyla, 60 genus and 228 species in the implant patients.

Both the health and implant samples showed a higher abundance value for *Fusobacterium nucleatum* and *Veillonella* species, which are the initial colonisers. The phyla *Firmicutes* is found to be the highest in both implant and healthy samples and the Genera *Prevetolla* was found have the highest abundance in both implant and healthy samples. At the species level *Porphyromonas pasteri* was observed to have the highest abundance in both implant and healthy samples.

A distinct salivary microbiome is found in implant samples when compared to healthy samples. Further studies need to be done to identify the role of these bacteria in periodontal health and implant patients.

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Annexures

ANNEXURE I- IRB REPORT

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003 - 06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOMSOEVER IT MAY CONCERN

Date: 30.1.2019

Place: Chennai.

From

The Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai- 600119.

The Dissertation topic titled **"MIROBIOME ANALYSIS OF WHOLE SALIVA USING NEXT GENERATION SEQUENCING TECHNOLOGY IN IMPLANT SUPPORTED PROSTHESIS 6 MONTHS POST LOADING"** Submitted by **DR. ASHA SRIKANTH,** has been approved by the Institutional Review Board of Ragas Dental College & Hospital.

Dr.N.S.Azhagarasan, M.D.S.
Member Secretary,
Institutional Ethical Board,
Ragas Dental College and Hospital,
Uthandi, Chennai - 600119

ANNEXURE II- PLAGIARISM REPORT

URKUND

Urkund Analysis Result

Analysed Document:	thesis toto.docx (D47313880)
Submitted:	1/28/2019 8:10:00 AM
Submitted By:	ashasrikanth261092@gmail.com
Significance:	6 %

Sources included in the report:

Review of Literature 2.docx (D47298311)
ENNET CYNTHIA JOHNS 1.docx (D47312844)
K.KAVIPRIYA -THESIS.docx (D47312847)
plagiarism check.docx (D34299138)
doc for analysis.docx (D34901232)
plagiarism.docx (D35097967)
https://mafiadoc.com/a-positive-correlation-between-occlusal-trauma-and-peri-implant-_598cc3401723ddcc692f031b.html

Instances where selected sources appear:

28

ANNEXURE III

CONSENT FORM

I ……S/o, w/o, d/o……
aged about …….years, Hindu/Christian/Muslim …… …….residing at ……do solemnly
And state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for

……
……

I was examined by Dr…….. and I was requested to do the following

1. Full mouth Plaque Score

2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in ……(language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorise the Doctor to proceed with further treatment or any other suitable alternative method for the study,

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu?.....) before me

Signature of the Doctor